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PURIFICATION OF POLIOMYELITIS VIRUSES

EXPERIMENTS ON MURINE AND HUMAN STRAINS

By

SVEN GARD

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To

My Father

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Preface.

The poliomyelitis research at the Institute of Physical Chemistry is sponsored by "Konung Gustaf V:s 80-Årsfond". I am deeply indepted to the Board of Directors of the Fund, and particularly to Professor Henning Waldenström for entrusting me with a share in these investigations.

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Uppsala, March 1943.

Sven Gard.

Introduction.

In 1909 Landsteiner and Popper (95) made the important discovery that infantile paralysis could be transferred to and maintained in passage in monkeys. In the field of experimental poliomyelitis research thereby opened, there followed a period of about thirty years, characterized by steady although slow progress. The ultimate aim of medical science is control of disease, and the research during this first epoch culminated in the elaboration of a number of therapeutic and prophylactic procedures — serum treatment, the Kolmer vaccination, zinc sulphate prophylaxis — with which great expectations were connected. When tested on a large scale in practice, however, they failed.

These failures aroused latent suspicions that the path hitherto followed might be a blind alley. Thus, in the later thirties an almost tumultuous development set in, which deserves to be termed revolution rather than evolution. The fundamentals of the prevailing theories were found at best unreliable, and characteristics of the virus-host interaction previously not recognized were now observed, necessitating a radical change of the conception of the whole problem. One of the principal reasons why the previous research had been lead astray was that most of the experimental work had been done in a heterologous host, the Rhesus monkey, and that it had not been recognized that the virus-host interaction in this animal in important respects differed from that in man.

When, about three years ago, the study of poliomyelitis was taken up at the Institute of Physical Chemistry, it was in the hope that it might prove possible to attack the problem from a new angle: to study the virus itself, its properties and reactions in vitro, as a basis of attempts at chemotherapy and chemoprophylaxis. *A priori* this scheme was not to be considered as only a remote possibility. As a matter of fact the giant molecules, to which class the viruses are

supposed to belong, seem to be in certain respects easier to study than molecules of a somewhat smaller order of magnitude.

It was, however, clearly realized that this program must involve extensive animal experiments as well. As a study of the virus in a model host had proved apt to lead to false conclusions, it was deemed advisable instead to study a model virus in its natural host. As model virus that of mouse encephalomyelitis (Theiler's virus) was close at hand. There must, in fact, be reason to expect that the remarkable similarities between the virus of human poliomyelitis and that of the mouse disease would not be limited to the observations already made, but that any further experience gained would be mutually relevant.

The first and fundamental item of the vast research program was the purification of the viruses in question. The present paper is a report on these experiments.

CHAPTER I.

The group of poliomyelitis viruses.

The avidity of a virus to certain tissues or organs and to certain host species has been a matter of much discussion. Attempts have been made to classify the viruses on the basis of their organotropism. At one end of the scale the pantropic viruses are equally capable of invading and multiplying in all kinds of tissues and organs. At the opposite end we find the monotropic viruses with affinity to one type of cells only. A similar variation is observed regarding possible hosts. Usually a virus has but one or a limited number of natural or homologous hosts. The virus-host interaction is usually conceived as an adaptation from the side of the virus to the habits of the host, guaranteeing the subsistence of the species. As a rule the virus can be experimentally transferred to and propagated in species other than the natural host, accessory or heterologous hosts. These often belong to species closely related to the natural hosts but sometimes the most whimsical constellations are observed. The combination of possible hosts is often a reliable characteristic of a given virus, by Doerr (29) called "Infektiositäts-Spektrum."

Sometimes one will find groups of closely related viruses, with the same organotropisms, causing identical symptoms and lesions, differing only with regard to host specificity. They have as a rule a narrow infectiosity spectrum. Such groups are for instance the rickettsiae, the pox and the influenza viruses.

With regard to the properties now mentioned the virus of poliomyelitis was for a long time considered almost unique. It was exquisitely neurotropic and with a narrowly limited tropism at that, directed towards certain functionally characterized elements of the central nervous system (CNS). It had an extremely narrow infectiosity spectrum, man being the only natural host and accessory hosts being found only among a very limited number of higher primates. Al-

though it was admitted that different strains of virus might exist, differing principally with regard to immunological properties, the existence of other viruses to be classfied in the same group was not recognized.

This conception had, however, to be revised. Already in 1912 Kling, Pettersson and Wernstedt (88) were able to show the existence of poliomyelitis virus in the intestinal contents removed at autopsy of cases of infantile paralysis. It was not, however, until Trask, Vignec and Paul (159) in 1938 had instituted the ether-treatment, thus considerably facilitating the examination of stools, that the significance of this observation was clearly realized. It has now been confirmed by numerous investigators [for references see Trask, Paul and Vignec (158)] that large quantities of virus are regularly excreted from the intestines, not only by patients with the typical paralytic form of the disease but by abortive cases and healthy contacts as well. Virus has also been found in the intestinal wall (133), and in mesenteric lymph nodes (87). Furthermore virus could be detected in sewage from urban districts, where cases of poliomyelitis had occured (116, 117, 85), partially under conditions to suggest that considerable quantities of virus were excreted in a population among which only a limited number of manifest cases had been observed. On the basis of this evidence one must assume that the virus is capable of multiplying not only in the central nervous system but also in the intestines.

Although experimental transmission of poliomyelitis to the ordinary laboratory animals had been without success in numerous attempts (61, 51), experiments to find new hosts have been repeated now and again. In 1939 Armstrong (1, 2) was the first to report success. In several experiments he was able to transfer the Lansing strain from monkeys to cotton rats (Sigmodon hispidus), and after passage through cotton rats to albinotic mice. The symptoms and histological lesions produced were indistinguishable from experimental poliomyelitis in monkeys. The virus retained its pathogenicity to monkeys even after prolonged serial passage in mice. Through neutralization and cross immunity tests the identity between the mouse passage strain and the original Lansing strain has been evidenced.

Transmission experiments of this kind can not be arbitrarily reproduced. Armstrong tested a number of strains, being successful only

with the Lansing strain. Negative results have also been reported by Toomey and Takacs (154), using 9 different strains, further by Hammon (59) and by Kramer and Mack (92), the latter authors using different procedures of inoculation known to increase the susceptibility of experimental animals to infection. Toomey and Takacs (155, 156) have later reported success with two strains of virus, after preceding treatment of the test animals with repeated subcutaneous injections of certain bacterial vaccines, a method previously used by Toomey to increase the susceptibility in monkeys to poliomyelitis.

Jungeblut and Sanders (76, 77, 78), working with the Sk strain, succeeded in producing in cotton rats an encephalitis of extremely acute course and high mortality rate. After passage through cotton rats the strain suddenly lost its pathogenicity to monkeys, producing only a subclinical infection with nondescript lesions. From cotton rats the virus could be passed to mice and maintained in serial passage, during which a certain fixation seemed to occur, the titer rising from 10^{-6} to 10^{-9}. At the same time the invasiveness increased, all tested routes of inoculation being equally successful. The mouse virus could easily be established in tissue culture of mouse embryo brain, the titer of the culture fluid reaching extremely high values. The fixed mouse strain produced encephalitis occasionally when inoculated into monkeys. The predominating symptoms were fever, coarse tremor, tonic-clonic convulsions and prostration. Surviving animals proved to be non-immune when tested with the original Sk strain. The mouse virus could be further transferred to guinea pigs, producing fever, paralysis of the hind legs and often death, the histological lesions resembling those of poliomyelitis. The strain could be maintained in brain to brain passages in guinea pigs but only for a limited number of generations, the infectivity gradually decreasing.

The last mentioned observations are rather confusing, differing definitely from the general experience of poliomyelitis virus on many important points. In interpreting the results one has to keep several possibilities in mind. The strain might have been inhomogeneous from the start, new components coming into preponderance when the experimental host was changed. Or latent viruses characteristic of the new hosts might have been picked up accidentally. This is known to occur rather frequently in rapid series of passages in small rodents (67, 150, 82), a procedure that Jungeblut and Sanders consider in-

strumental in establishing the infection in cotton rats. On the other hand sudden changes, the character of mutations, might occur during passage in accessory hosts, even of a pure strain of virus. Definite conclusions as to the significance of Jungeblut and Sander's observations must be postponed until further investigations (for instance filtration endpoint determinations) have revealed the true nature of their passage strain.

For identification of the virus of poliomyelitis it has been usual to rely upon the following criteria: (a) production in monkeys of the characteristic clinical picture with the typical histological lesions, (b) successful passage to another monkey, and (c) the virus should be non-infectious to the ordinary laboratory animals. The third criterion must be considered insignificant after the observations of Armstrong, and the second is of little value since it is well known that most strains are adaptable to monkeys only with difficulty or not at all. The remaining one is in fact of a secondary nature, referring exclusively to the reactions of the host. As a matter of fact it has until now been impossible to detect any direct evidence of the presence of the virus in infected tissue in the form of inclusion bodies or the like. In most virus infections reliable information about the nature of the virus can be gained through serological reactions. In the case of poliomyelitis this is hardly feasible. The true nature of the neutralization in vitro, if specific or not, has been a matter of discussion and as to other reactions they have failed completely (123).

On the other hand, the state of affairs indicated above necessitates great caution in evaluating the observations by Armstrong and Jungeblut and Sanders, since there are known to exist at least two spontaneous virus diseases in different animal species, showing all the clinical and histological characteristics of poliomyelitis. These diseases are known as the Teschen disease of swine and the spontaneous encephalomyelitis of mice (Theiler's disease).

The Teschen disease (Bohemian disease, infectious swine paralysis Encephalomyelitis enzootica suum) was first observed in epizootic form and recognized as a clinically and etiologically distinguished disease in the year 1929 in Teschen (Czechoslovakia). It has since then remained enzootic in this part of Europe and spread slowly southwards and westwards. In 1938 cases occurred in Bavaria and

Austria, in 1939 in Switzerland and in 1940 in the neighbourhood of Leipzig.

Klobouk (89) made an extensive study of the disease and identified the infectious agent as a filterable virus that could be propagated in serial intracerebral passage in swine. The literature on the subject is quite comprehensive and for a more exhaustive review reference might be given to articles by Lentz (98) and Weidlich (160).

Clinically an acute, a subacute, and a chronic form of the disease are distinguishable (146). The acute disease is most common in sucklings or animals just weaned. After a short stage of fever and, occasionally, of vomiting, alarming encephalitic symptoms appear: hyperirritability, sometimes coma, convulsions of different groups of muscles preferably of the head, nystagmus, and manège movements. Skin hypersensitivity is common. In most cases death occurs within 3 or 4 days.

In the subacute and chronic forms flaccid paralysis of the limbs is the predominating symptom. Sometimes spastic paralysis is observed. The hind quarters are most often involved, but isolated paralysis of other muscles has been observed. As the "simple form" Diernhofer (28) describes an ascending paralysis of the Landry type. The mortality rate is about 20 p. c. A considerable number of the surviving animals show atrophy of the paralysed muscles, but sometimes complete recovery is attained.

At autopsy no gross lesions are observed. On microscopical examination lesions are found in the CNS, consisting of ganglion cell degeneration with neuronophagia, scattered round cell infiltration and glial reaction, and finally, perivascular round cell cuffing (89, 6, 90). Meningitis is usually not observed. The lesions are confined to the gray matter, preferably of the anterior horns of the cord and the basal nuclei of the brain. According to Kment (90), however, a leptomeningitis and infiltration of the cerebellum with distinct alterations of the Purkinje cells should regularly occur.

The disease can be transferred experimentally through intracerebral injection of emulsified cord. The incubation time amounts to one to four weeks and the symptoms and lesions are identical with those of the spontaneous disease. Young animals are regularly susceptible to experimental infection, whereas a large percentage of old animals are resistant. Transmission by the nasal route is possible although with far less regularity; subcutaneous and intravenous

inoculation succeeds only occasionally. It has not been possible to transfer the disease to mice, rats, guinea pigs, rabbits, fowls, sheep, or calves (89, 28).

About the portal of entry and the excretion of the virus and the natural mode of transmission of the disease nothing is definitely known. On the basis of successful feeding experiments, Klobouk suggested the alimentary route. Diernhofer, on the other hand, advocates the nasal mucosa as portal of entry. Rats have been suspected to be intermediate hosts of the virus (28).

Several authors (136, 90, 40) have emphasized the similarities between the Teschen disease and the human poliomyelitis.

Theiler's disease. The first description was given by Theiler (147) in 1934. In a later communication (148) he presented details about the disease and its causative agent, shown to be a filterable virus. He emphasized in this paper the similarities between this disease of mice and the human poliomyelitis. Furthermore an extensive study of the disease and its causal agent was reported by Theiler and Gard (150, 151).

The main feature of the clinical picture is flaccid paralysis of the limbs, principally the hind legs, sometimes of the ascending type, terminating in respiratory paralysis and death. Sometimes the nervous disturbances are very mild and complete recovery is attained. More often, however, paralysis leads to muscular atrophy and contractures. With the exception of very advanced cases the animals retain their natural liveliness. Their fur is sleek, their appetite normal.

At autopsy no gross changes are found. On microscopical examination (110) lesions can be found only in the central nervous system. They consist of neuronal disintegration with the typical picture of neuronophagia, further perivascular round cell cuffing, often extending to the meninges, and a scattered round cell infiltration and glial reaction in the most heavily invaded areas. In late stages of repair nothing is to be seen except a pronounced scarcity of motor ganglion cells. Preferred areas are the anterior horns of the lumbar and cervical cord, although involvement of higher centers, especially of medulla and pons regularly occurs, at least after intracerebral infection. (Plate I.)

The attack rate of the spontaneous disease is very low. Theiler originally estimated it to one or two per thousand. Sabin and Olitsky

(132) arrived at similar figures. For all we know even this moderate figure might be too high. Olitsky (107) has later observed a series of more than 5 000 mice with no cases. The present writer observed during 30 months a total of about 15 000 animals in which period 5 spontaneous cases of the disease were discovered, four of these occurring almost simultaneously during an outbreak in the colony of mouse typhoid (caused by S. enteritidis).[1]

It is generally young mice that contract the disease spontaneously. All instances known to the present writer concern animals of from 4 to 8 weeks of age.

In contrast to the low attack rate the disease is geographically widely distributed. Besides in the U. S. it has been observed in Germany (53), in Japan (73), at the Rockefeller stations in Brazil and Uganda (Theiler pers. comm.), in Palestine (Kligler pers. comm.), and in Sweden (own observation).

The disease can be maintained indefinitely in serial intracerebral passages of brain or spinal cord. Under experimental conditions the infectious agent displays an exquisite neurotropism. Direct contact with nerve elements is the only mode of infection to yield success with any degree of regularity. With highly virulent strains of virus the intranasal and intraperitoneal routes might prove successful if very large amounts of virus are inoculated. The highest concentration of virus following infection is usually found in the spinal cord, although at the height of disease the brain and periferal nerves might be infective as well, whereas organs and tissues outside the nervous system seem to be virus free. There is furthermore no indication of an elimination of the infectious agent from the central nervous system by way of excretion.

Recovery from spontaneous or experimentally induced disease is regularly accompanied by a solid immunity to subsequent infection. In cross immunization experiments it has been possible to show that all strains of mouse encephalomyelitis virus are very closely related,

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[1] This is not the place to discuss the eventual epidemiological significance of this observation. About the pathogenesis of the spontaneous infection of the central nervous system very little if anything is known. In this connection it might be mentioned that Olitsky (107) attempted to induce infection from the intestines by means of a rather drastic treatment with croton oil, resulting in almost complete loss of the mucous membranes, yet without success.

although slight immunological differences seem to exist. It has been observed that animals recovered from mouse encephalomyelitis were resistant to subsequent infection with Armstrong's Lansing strain (150).

With age the resistance to experimental infection increases although a level of immunity comparable to that attained after actual disease is never reached. Signs of increased resistance are lengthening of the incubation period, milder symptoms and lower mortality rate. Real immunity in apparently normal mice has been observed only once (own observation). In this case it was possible to isolate from the central nervous system a strain of mouse encephalomyelitis virus, clearly different from that with which the test inoculation had been performed. This unique case must therefore be regarded as one of immunity following naturally acquired infection with complete recovery.

The wild mouse (Mus musculus) is as susceptible to infection with mouse encephalomyelitis virus as the albinotic variant. A number of other rodents tested proved to be refractory, viz. field voles, deer mice (Peromyscus), white rats, guinea pigs and rabbits. It has been shown, however, that at least one strain is pathogenic for the cotton rat (150). Attempts to transfer the disease to monkeys have been without success.

The infectious agent passes ordinary bacterial filters readily. Theiler and Gard (150) determined the filtration endpoint to between 35 and 27 mμ. With exactly the same technique Theiler and Bauer (149) had previously found a filtration endpoint for human poliomyelitis virus betwen 35 and 30 mμ. The virus of mouse encephalomyelitis is rapidly destroyed at temperatures above 50° C, but shows a considerable stability at room and ice box temperatures. Stored in 50 p. c. glycerin at + 4° C infected brain and cord retain the infectivity for years. An aqueous solution was kept under ether in the ice box for 100 days without apparent loss of activity. Moderate concentrations of acetone are well tolerated whereas ethyl alcohol has a definite inactivating effect. The virus can be precipitated with ammonium sulfate and redissolved without loss of activity. Desiccation from the frozen state on the other hand, frequently used as a method for conservation of the viability of different viruses and bacteria, is deleterious to the virus of mouse encephalomyelitis as it is to human poliomyelitis virus (51). Exposure to the air seems to have little

influence on the stability of the virus, but oxygen in the nascent state has a definite inactivating effect.

In 1939 Olitsky (106) made the important observation that encephalomyelitis virus could be recovered from the intestinal contents of apparently healthy mice. Later studies on this subject have revealed the following facts (107, 108, 151). In sucklings the virus has never been found even if the mother was a carrier or was infected experimentally. At the age of weaning from two to four weeks, virus can occasionally be found in the intestines. From then on the frequency of positive findings rapidly increases so that at the age of six weeks practically 100 p. c. of stock mice carry the virus in their guts and excrete it with their feces. They remain carriers for shorter or longer periods of time, in some instances probably for life.

Virus can be recovered from feces, from the intestinal contents, from the intestinal wall, frequently from mesenteric lymph nodes, occasionally from the stomach contents but not from the stomach wall, never from oesophagus nor from any part of the head and not from any interior organs. The actual site of infection and multiplication of virus is not definitely known. After rinsing of the gut in running tapwater with gentle rubbing of the mucous membrane it will be found on histological examination that considerable quantities of intestinal contents are still retained in the crypts and between the villi of the mucosa. From intestinal wall thus treated, virus is easily recovered. When the guts are washed more energetically by repeated shaking with water or physiological saline solution, the mucous membrane will be severely injured, most villi completely destroyed and only the basal parts of the epithelium left. In spite of this, contents are likely to be found still clinging to remnants of the mucous membrane. Peyer's patches are usually well preserved. Such preparations have generally a low virus content and are occasionally virus free. It seems probable, therefore, that the virus is to be found either in the superficial parts of the mucous membrane or extracellularly on the surface of the epithelium. The presence of virus in the lymph nodes might be interpreted as the result of absorption rather than as evidence of invasiveness.

Furthermore Olitsky has been able to demonstrate the presence of virus in wild mice, trapped in the laboratory, but so far not in wild mice caught at some distance from places where stock mice were housed (111).

Human, murine and porcine poliomyelitis.

Students of poliomyelitis have long been used to looking upon this virus as something absolutely unique, in a way of speaking as a gruesome privilege of the human race. From a scientific point of view this attitude is of course absurd. The existence of coordinated types of the virus with different host specificity might be denied but not until the facts have been taken into unbiased consideration. If on the other hand relationships are found, close enough to support the conception of different strains as variants or types of the same species, it would be only logical to express this view in the choice of nomenclature.

Iguchi (73) suggested the name mouse poliomyelitis for Theiler's disease, and this terminology was adopted by Olitsky. From certain quarters, however, objections have been raised against the premature use of this name, especially on behalf of the confusion brought about by Jungeblut and Sander's observations. The problem of the systematic position of Theiler's virus has undoubtedly gained in importance after the establishment of murine strains of the human virus. This fact might justify an attempt to review the present state of the question.

Of the two viruses, that might be classified with the poliomyelitis virus, that of mouse encephalomyelitis has been by far the most extensively studied and an analysis of the situation must be concerned principally with this virus. Available data on the Teschen disease quoted from the literature will however be included in the following survey, which is presented in tabular form in order to avoid reiterations. (Table I.)

A few items of the table need some further elucidation.

The seasonal distribution in poliomyelitis is very characteristic, the peak of the frequency curve almost invariably to be found in the fall. This is particularly conspicuous in the cold and temperate climates of the northern as well as the southern hemisphere. In subtropical zones the distribution is much smoother, and in the tropics seasonal differences are hardly noticeable. A similar frequency distribution of the spontaneous mouse disease has not been observed, with the possible exception mentioned on page 11, when in the course of one year a total of four cases was observed in the mouse colony in question, all of them within a period of two weeks in August and

Table I.

Comparison between human poliomyelitis, mouse encephalomyelitis, and the Teschen disease.

	Human poliomyelitis	Mouse encephalomyelitis	Teschen disease
Clinical picture	Preparalytic stage of fever and meningeal symptoms.	Not known	Fever and meningeal symptoms.
	Paralytic stage: Flaccid paralysis, mainly of the limbs.	The same	The same
	The ascending type of paralysis common.	The same	The same
	Cerebral forms exist, with ataxia and spastic symptoms.	Cerebral forms exist, tonic-clonic convulsions main symptoms.	Cerebral forms common in young animals. Spastic symptoms.
	Non-paralytic forms exist.	The same	The same
	Incubation period of experimental disease usually 10—14 days.	The same	The same
Pathology	No gross lesions.	The same	The same
	Histological lesions confined to the CNS.	The same	The same
	Primary process: neuronal disintegration, neuronophagia.	The same	The same
	Secondary features: glial reaction, round cell infiltration, perivascular cuffing.	The same	The same
	Principal site of lesions: anterior horns of the spinal cord.	The same	The same

Table I cont.

	Human poliomyelitis	Mouse encephalomyelitis	Teschen disease
Epidemiology	Annual morbidity rate about 1:10 000. Epidemic attack rate sometimes up to 10 p. c.	Attack rate about 1:5 000.	Epizootic attack rate up to 50 p. c.
	Age distribution: prevalence of children and young adults	Prevalence of young mice.	Prevalence of young animals.
	In temperate climates characteristic seasonal distribution.	Seasonal distribution even.	Cold-wet seasons preferred.
	Portal of entry of virus not definitely known, probably alimentary tract, principally intestines.	Not known. Probably intestines.	Not known
	Elimination of virus: principally feces.	The same	Not known
	True contact cases probably rare.	The same	The same
Immunology	Reinfections are very rare.	Solid immunity after recovery from spontaneous or experimental infection	The same
	Immunologically different strains of virus seem to exist.	The same	Not known
	Neutralizing substances appear in the blood with age. Apparently no connection with clinical disease.	The same	Not known
	Attempts at precipitin reaction and complement fixation unsuccessful	The same	Not made

Table I cont.

	Human poliomyelitis	Mouse encephalomyelitis	Teschen disease
Distribution of virus	In clinically manifest cases: Virus in the CNS but no other tissues except the intestinal wall and sometimes mesenteric lymphnodes.	The same	Virus in the CNS. Intestines with contents not examined.
	Healthy carriers harbour virus in the intestines. To what extent not known.	Carrier state is the rule.	Not known
Properties of the virus	Infectiosity spectrum narrow: man, certain primates, occasionally cotton rat and mouse.	Infectiosity spectrum narrow: albinotic mouse, gray mouse, occasionally cotton rat.	Ordinary laboratory animals refractory.
	Under experimental conditions strictly neurotropic.	The same	The same
	Generally not cultivable in tissue culture.	The same	No attempts
	Filtration endpoint: 30 mμ.	Filtration endpoint: 30 mμ.	Not studied
	Remarkable resistance in vitro.	The same	Not studied

September. The domestication of the animals, however, makes a direct comparison meaningless. From a climatic point of view the domesticated mice might be said to live under conditions close to those prevailing in the tropics.

The portal of entry of the virus of human poliomyelitis is not definitely known. From experiments on Rhesus monkeys, which can be "naturally" infected only through nasal instillations, it was generally assumed that the olfactory region of the nasal mucous membrane was the main portal of entry, and that the disease was

transmitted through droplet infection. In Rhesus monkeys infected intranasally characteristic microscopic lesions are regularly produced in the olfactory bulbs (131). In human cases of poliomyelitis, however, lesions seldom occur in the olfactory region of the brain and particularly not in the olfactory bulbs (54, 60, 62, 65, 129). Howe and Bodian (70) analysed the spread of virus and distribution of lesions in the CNS after experimental infection of chimpanzees along different routes. They found that the localization of lesions often showed a characteristic pattern, owing to the mode of infection. Examining a number of human cases from this point of view (71), they found a distribution indicating that the virus had gained access to the CNS through the spinal roots, probably from the intestines, although the cutaneous route could not be excluded. In one case it was possible to locate the portal of entry to the mouth and throat. Earlier observations had called attention to the possible connection between tonsillectomy (3, 38) or tooth extraction (45) and subsequently appearing poliomyelitis, likewise pointing towards the alimentary rather than the respiratory tract as portal of entry.

These observations combined with the fact that virus can be recovered only occasionally and in small quantities from nasal washings[1] but almost regularly and in large quantities from stools, decidedly favour the theory that the entry point of the virus is to be found in the alimentary tract. As a further indirect evidence of the alimentary mode of transmission versus droplet infection may be mentioned that Rhesus monkeys, which succumb to intranasal but are refractory to alimentary infection never contract the disease spontaneously even if caged with experimentally infected animals, whereas spontaneous infection of chimpanzees, which are readily susceptible to infection from the alimentary tract has been observed (104). Although definite evidence in the form of details about the mechanism of invasion is still lacking, the recent development of our knowledge on this point strongly supports Kling's theory of poliomyelitis as a waterborne disease, according to which alimentary infection was postulated (84).

[1] Preparations termed nasal washings in the literature have as a rule been combined nasal and throat washings. To the writer's knowledge no serious attempts have been made to avoid contamination with throat discharge. The virus found in preparations of that kind must, therefore, not necessarily originate from the nasal mucous membrane.

In the case of mouse encephalomyelitis the present data tend to show that an apparently symptomless infection of the intestines is the normal course of the disease, while the "typical" paralytic form has to be regarded as a rare accident. Since mice are habitually coprophageous, it is natural to assume alimentary transmission of the infection. Attempts to establish enteric infection and carrier state in sucklings by feeding with active virus have failed, however. Sometimes the mice thus treated succumb in an invasion of the CNS, while in the remainder the virus is rapidly eliminated from the intestines, usually in the course of 24 hours. In suckling mice, therefore, some condition necessary for the development of carrier state, even temporary, seems not to be fulfilled. It is close at hand to connect the act of weaning and the changes of the intestinal bacterial flora, thereby induced, with the apparent change in disposition to enteric infection. On this point, however, no significant observations have been made so far.

As to the mechanism of invasion of the CNS and the point of entry nothing is definitely known. One case was examined from this point of view by the present writer. In serial sections of the CNS it was not possible to detect any lesions in the olfactory bulbs or at any level above the oblongata. In analogy with the observations by Howe and Bodian this would indicate that the virus had gained access to the CNS through the spinal roots, pointing towards the intestines as portal of entry.

The theory of transmission of poliomyelitis through personal contact was previously generally accepted and is still advocated in spite of the fact that secondary cases explainable through direct contact are extremely rare. One had therefore to assume indirect contact through symptom-free intermediate carriers as the normal mode of transmission. The comparatively rare institutional and family outbreaks of the disease were usually quoted as instances of direct contact infection. The time interval between family cases is generally less than a week, which fact led to the assumption of a very short incubation period, hardly in line with the experience from experimental poliomyelitis. An analysis of some institutional and family epidemics in Sweden showed, however, that these could be reasonably explained as cases of simultaneous infection with variation in the length of the incubation period within the limits usually observed in experimental poliomyelitis, another indirect evidence of alimentary transmission (45).

The neutralization reaction in poliomyelitis has been subjected to extensive study. The comprehensive literature in this field will not be reviewed in this connection; the experimental results only may be summarized as follows. The frequency of positive neutralization reaction increases with age. The frequency of positive reactions in convalescents is usually slightly below that of normals of the same age. The neutralizing titer of pooled convalescent serum is usually lower than that of pooled normal serum. A positive reaction affords no reliable protection against infection. Trask and Paul actually describe a case, where the reaction was positive on the third day of illness and after three weeks, but negative on a later occasion (157). There is, thus, little evidence to show any connection between the typical disease and the appearance of neutralizing substances in the blood. Whether this process is to be regarded as an expression of developing maturity resistance ("autarcesis", Aycock) or as the only visible response to repeated enteric infections cannot be settled at present.

Regarding mouse encephalomyelitis Olitsky has shown that serum from normal adult mice is capable of neutralizing the virus, and that no differences in this respect could be demonstrated between normal and convalescent serum (109).

* * *

From the data in table I it is evident that the viruses of human poliomyelitis and mouse encephalomyelitis are closely related. In the writer's opinion particular weight should be attached to the identity of host reaction against infection and to the identity of particle size of the viruses.

Typical neuronophagias are considered pathognomonic for poliomyelitis. They are seldom observed in other virus infections of the CNS but they are always present in poliomyelitis, at least in the early stages of the disease. The micrographs of plate I reveal the remarkable similarity of histopathology in mouse encephalomyelitis and experimental poliomyelitis in monkeys.

This feature refers, however, primarily to the host. More significance as evidence of a true relationship must be attached to the results of the filtration endpoint determinations. With the technique introduced by Elford (32, 34) easily reproducible results can be ob-

tained. The particle size of a certain virus seems to be a fairly constant quality, at least no appreciable variation can be detected by present methods. The filtration endpoint must therefore be regarded as an important characteristic of a virus. For instance two viruses with similar clinical manifestations as those of vesicular stomatitis and of foot-and-mouth disease can easily be distinguished by means of ultrafiltration, one having an endpoint of 120, the other of 25 mμ.

In a filtration experiment on poliomyelitis virus Theiler and Bauer (149) found that the virus passed a 35 mμ membrane in one out of 4 tests, but had negative results with a 30 mμ membrane in 6 tests. Elford, Galloway and Perdrau (35) found that the virus passed readily 40 mμ membranes, and in one instance out of 5 tests a 27 mμ membrane filtrate apparently produced immunity in the test animal, although no clinical infection was obtained. As positive evidence this result must be considered questionable and 30 mμ as retaining pore diameter would be a safer estimate than the value of 25 mμ, assumed by Elford. As already mentioned, Theiler and Gard (150) found that the mouse encephalomyelitis virus readily passed 35 mμ membranes, but was retained by a 27 mμ membrane. In this case, too, an estimate of 30 mμ for the filtration endpoint is reasonable. According to Elford's formula the particle diameter of both viruses can be calculated to 10 to 15 mμ (33).

In the range of filtration endpoints between 25 and 35 mμ no other neurotropic viruses are represented, and only one additional animal virus, viz. that of foot-and-mouth disease. Under these circumstances identity of particle size must be considered as strong evidence of close relationship.

All facts considered, there remains in the writer's opinion no essential distinguishing differences between the viruses discussed except the species specificity. As the situation is, they must be conceived as variants or types of the same species until evidence to the contrary can be produced. Consequently they should be classified together and uniformly denominated. From several points of view the name poliomyelitis virus is most unsatisfactory. It is, however, established by usage, and until the question of virological nomenclature has been brought out of its present state of confusion, this name has to be accepted. In the following, therefore, the respective diseases will be called human, murine or mouse, and porcine or swine poliomyelitis,

and the causal agents referred to as poliomyelitis viruses of the human, murine, and porcine types.

The experiments to be reported were to a certain extent based upon this conception as a working hypothesis. In this study the mouse disease and its virus served as model of the human poliomyelitis. All methods were accordingly worked out in detail in experiments on the mouse virus and then simply applied to human strains. As will be shown in the following this simplification was justified by the results.

CHAPTER II.

Material and general methods.

Strains of virus.

The estimates of the particle size of poliomyelitis viruses, based upon ultrafiltration data, has placed them among the smallest hitherto known. From a gravimetric point of view one had therefore to expect extremely small yields in purification experiments, so much more so as the infectivity of virus containing tissue usually remains on a low level, the titer seldom exceeding 10^{-4}. It was consequently considered important to use as highly virulent strains as possible. For the experiments on mouse poliomyelitis, therefore, the FA strain was chosen. As this strain on several points differs from the general description given in the preceding chapter it will be described in detail.

The FA strain was accidentally picked up by Theiler during serial passages in mice of the neurotropic French strain of yellow fever virus (150). It was observed that the incubation period, for that particular yellow fever strain regularly 6—7 days, from a certain passage onwards suddenly decreased to 4 days, while the symptoms indicating an acute encephalitis were largely unchanged. In serological tests the new passage strain was not neutralized by anti-yellow fever serum nor by anti-choriomeningitis serum. Histological examination revealed that it was probably a mouse poliomyelitis virus with pronounced encephalitogenic properties. During serial passage the strain has become "fixed," the incubation period after intracerebral inoculation of a 10 p. c. brain suspension being 2—3 days.

In the clinical appearance of infection three stages can be distinguished. The first stage is very vague in character and not always noticeable. The animals display a certain nervousness and irritation without distinct symptoms. It might be analogous to the fever and meningeal irritation preceding the onset of paralysis in human and

monkey poliomyelitis. It is usually observed only in animals infected with a medium amount of virus. During this stage the maximum concentration of virus in the brain is not yet attained.

The second stage is characterized by temporary attacks of tonic-clonic convulsions. These fits are usually provoked by mechanical, acoustical or optical irritaments but sometimes they seem to appear spontaneously. Spinning the animal by the tail seems to be a reliable way to incite them. They are usually of short duration, about 30 seconds. After an attack a refractory period sets in, during which it is impossible to incite a new fit. A typical attack begins with twitches of the eyelids and jerks of the head, the back is stretched, sometimes the animals turn over backwards, and characteristic clonic movements of the fore limbs set in. In laboratory slang this condition is called "jitter-bug disease," which is an exellent description of the behaviour of the animals. (Plate I) Sometimes the mere contact with the floor incites spastic movements of the legs and the animals make a series of grasshopper-like jumps of considerable height and length. A fit often terminates in a tonic convulsion, during which the back is extremely stretched, the fore legs pressed against the chest and the hind legs stretched backwards. Respiration temporarily ceases and sometimes death occurs.

If the animal survives the early attacks, the third stage enters. Symptoms are now permanent. The animals look obviously sick. The fur is ruffled. The back is hunched. Their appetite is lost. Motor disturbances are common: convulsions, spastic or flaccid paralysis. In the males permanent erection is observed almost invariably.

Sometimes, preferably in old animals, no encephalitic symptoms appear. Then the disease takes the classical course with flaccid paralysis as the main symptom. In this case, however, the fore legs are more often involved than the hind legs, and the general condition of the animals usually more affected than in the classical disease.

The mortality rate is practically 100 p. c. Sometimes, however, old animals survive an experimental infection.

The clinical picture is to a certain extent dependent upon the route of infection. (a) After intracerebral inoculation the incubation period varies with the amount of virus injected from 2 to 20 days. The course of the disease is as described above, encephalitis being the principal feature. (b) Infection by the intranasal route is possible, when large amounts of virus are used. After an almost constant

incubation period of 15 days, the animals develop an encephalitis. The mortality rate among animals showing symptoms is practically 100 p. c. (c) After intraperitoneal inoculation of large amounts of virus a certain percentage of the animals show the classical symptoms, i. e. flaccid paralysis, usually of the ascending type. Encephalitic symptoms are rare. The mortality rate is definitely lower, usually about 50 p. c. The incubation period is constant and about 10 days. — The susceptibility of the mouse to infection by the intracerebral route is about 100 000 times as high as by the intranasal and about 10 million times as high as by the intraperitoneal route.

The lesions are identical with those of the classical disease: disintegration of ganglion cells with neuronophagia, glial reaction and round cell infiltration, mainly perivascularly. The most intense reaction is found in the basal parts of the brain and the brain stem, the cortex is regularly affected, although less intensely. (Plate I) In the hyperacute cases usually no lesions are found in the cord. Cases of longer duration, however, show typical lesions in the anterior horns.

The maximum concentration of virus is found where the lesions are most pronounced, i. e. in the brain. During the second stage of the disease the brain is regularly infective in the concentration 10^{-7}, when inoculated intracerebrally in 0.02 ml amounts. The infectivity of the spinal cord is definitely lower, usually by two or three powers of ten, except after intraperitoneal inoculation when the same maximum concentration is reached.

In ultrafiltration and cross immunization experiments the FA strain has been identified by Theiler and Gard as a strain of mouse poliomyelitis virus.

When used in the experiments to be described in the sequel the strain had been maintained in brain to brain passages through more than 100 generations. As a rule each experiment was set up with a fresh passage of virus, the entire series of experiments, therefore, being extended over further about 50 passages. No appreciable changes of the virulence or any other characteristics of the strain were observed during the period in question.

In a few experiments the less virulent strain UF I was used. This strain originated from an apparently healthy mouse, 6 weeks of age and was isolated from the ether-treated intestinal contents of the animal. It has been maintained in cord to brain passages. After

intracerebral inoculations of 0.02 ml of a 10 p. c. cord suspension, the attack rate has been almost invariably 100 p. c., and the incubation period between 10 and 15 days. The clinical picture has always been of the classical type without any signs of encephalitis. The histological lesions produced have been typical.

No experiments on passage strains of human poliomyelitis were carried out.

Sources of virus.

In several cases allantoic membranes, amniotic fluid or tissue from infected chick embryos has served as an excellent starting material in purification experiments on account of their high virus content. As will be reported later in detail, it is possible to propagate the mouse poliomyelitis virus in the growing chick. The infectivity of the embryonic tissue remains, however, on a very moderate level. For purification purposes, therefore, this source of material offers no advantages.

Tissue culture fluid, being practically free from coarse tissue debris and high molecular proteins, would be an ideal starting material. In attempts to cultivate human poliomyelitis virus on chick embryo brain tissue culture, success was reported by Gildemeister (52) and later confirmingly by Pauli (118). Sabin and Olitsky (130) were unable to confirm Gildemeister's observation but found that their strain of virus could be propagated in tissue culture of human embryo brain. Recently Jungeblut and Sanders (76) found the murine Sk strain easily cultivable on mouse embryo brain tissue culture.

In spite of previous failures by the present author (unpublished data), numerous experiments were set up in attempts to establish mouse poliomyelitis virus in tissue culture. As all efforts towards that end remained unsuccessful the experiments will be only briefly summarized. The tissues tested were mouse embryo brain, whole mouse embryo, chick embryo brain and whole chick. The culture fluid consisted of Tyrode's solution, with or without serum. Human, monkey and mouse serum as well as serum ultra filtrate (135) were used. The cultures were incubated at 37 and 18° C, aerobically as well as anaerobically. The virus remained active in these cultures for considerable periods of time, especially at room temperature. In no instance, however, an increase in activity could be detected. When subcultures

were made at intervals of five or ten days, the virus could seldom be traced beyond the fifth subculture.

The choice of source of virus was, therefore, restricted to infected brain and cord, or feces. In the course of the investigation observations were made, suggesting the possibility that differences might exist between virus isolated from the CNS and virus from the intestines of the same host. Until this question has been definitely settled, it seems reasonable to make a distinction between virus preparations derived from different sources. It must be pointed out, however, that the expressions neurovirus and intestinal virus, which will be employed in the sequel, merely indicate the origin of the virus but do not necessarily imply fundamental differences.

In purification experiments on neurovirus from mice usually batches of 500 brains were prepared. The animals were killed with chloroform as soon as definite symptoms had appeared, the brains removed aseptically and stored in 50 p. c. glycerin in the cold until used. For collection of feces the animals were caged on sifted sawdust in large boxes. Every two or three days the litter was collected and resifted under gentle rubbing and pressure. Practically only feces remained on the sieve, while sawdust, crumbs of bread and the like passed the meshes.

Purification experiments on human virus were likewise carried out on nervous tissue and intestinal contents. At a number of autopsies on typical cases of poliomyelitis, brain and spinal cord were removed. Attempts to maintain bacteriological sterility were not made. The material collected was stored in 50 p. c. glycerin in the cold until used. Brain and spinal cord from one lethal case of ventricle cancer and from a man who died from injuries suffered in an accident served as control material.

As source of intestinal virus colon washings and clysters were used. The material originated from hospitalized typical cases of poliomyelitis. Washings of from one to two liters were collected, sent to the laboratory, and treated immediately. The samples were taken preferably during the first two weeks after onset of the disease. "Normal" controls, colon washings from a number of cases without symptoms from the central nervous system, were obtained from the Medical School Hospital of the University of Upsala. Finally samples of feces from a number of healthy contacts were secured.

In an appendix on page 163 the hospital record numbers of all cases included in this study are filed.

Inoculation technique and diagnosis.

In the infection experiments the intracerebral route was used throughout. The test material, consisting of different fractions from the purification process, was usually not bacteriologically sterile. On account of unavoidable losses of activity filtration could not be performed. On some occasions the solutions were treated with ether before inoculation but when this was not practicable even such precautions had to be omitted and the material was inoculated without further treatment. In spite of this, intercurrent infections to be ascribed to bacterial contamination of the inoculum were never encountered.

All inoculations were carried out under ether anesthesia. For anesthetization of mice the following simple device was employed. From a gasometer air was bubbled through a flask containing water and ether, and immersed in a waterbath of 20 to 25° C. From this flask the air was led to the bottom of a glass jar with an inserted wire net bottom, on which the animals were kept. By means of this arrangement it was possible to apply a mild and even anesthesia simultaneously to a large number of mice.

For the inoculation of mice tuberculin syringes and 20 gauge needles were used. Without previous shaving or surface disinfection of the skin the needle was inserted through the right parietal bone, and an inoculum of 0.02 ml deposited at a depth of about 3 mm below the brain surface. On monkeys the skin was shaved and disinfected with alcohol over the left temporal bone, a slit made with a knife and the bone trepanned, the 12 gauge needle of a syringe inserted, and an inoculum of 0.5 ml deposited at a depth of about 1 cm in the temporal lobe.

The diagnosis of mouse poliomyelitis was based principally upon the clinical manifestations. In tests of crucial character, however, sections were prepared and examined microscopically. In the monkeys the rectal temperature was recorded in daily measurements and the motility carefully controlled while the animals were being exercised in a special large cage. In case of typical symptoms the animals were sacrificed after the fall of the temperature, and autopsy performed. Sections of the CNS were regularly prepared and examined microscopically. As a rule control of bacteriological sterility was carried out only when the presence of gross lesions suggested bacterial infection.

Stock animals.

Already now it may be pointed out that in the kind of experiments to be reported the breed of test animals used is of the utmost importance for the uniformity of the results. This was clearly realized from the start, and attempts were made to secure a pure breed of mice. No such animals were, however, available. There was finally obtained a mixed breed, descent not known, but very uniform of appearance and size and in good condition. This stock has since been maintained through inbreeding.

As the possible presence among the test animals of latent virus infections, that might interfere with the experimental results, is a factor of great importance, several attempts were made to clear up the situation in this respect. Various methods of provocation were tested now and again with special attention to the viruses of choriomeningitis, ectromelia and mouse influenza. No latent infections could be discovered.

Each summer, however, beginning in July or August and extending over September and part of October, a sharp increase in the mortality rate of the stock animals was observed, concerning principally sucklings and females during the first two weeks after delivery. Sometimes the animals were found dead without having previously shown any signs of disease. As a rule, however, they were in a bad condition for one or two days preceding death, with loss of appetite and emaciation. With the exception of an occasional diarrhoea usually no local symptoms were observed. Sometimes, however, convulsions and spastic or flaccid paralysis occurred, not unlike symptoms of mouse poliomyelitis. Gross lesions when found at autopsy, were restricted to the skeleton, and consisted of a certain porosity of the bones and frequently proliferative excrescences of the ribs and the vertebrae. Numerous sections of the CNS were carefully examined for the presence of poliomyelitic lesions. Now and again degeneration of ganglion cells in the spinal cord was observed but never any typical lesions. Passage experiments with various organs employing different routes of inoculation remained unsuccessful. Aerobic and anerobic cultures in plain broth and on blood agar from spleen, liver and lymph nodes were consistently negative.

Animals of this stock were sold to two other laboratories and used for breeding purposes. In none of these places the same calamity

was encountered. Although no dietary changes were instituted the disease was finally interpreted as an acute vitamine deficiency, possibly on the basis of gastro-intestinal disturbances.

During that part of the year when the conditions just described were prevalent in the animal rooms, experimental work became hazardous, partly because the numerous deaths among the test animals could not always be accounted for and partly because the mice during that period showed an increased resistance against infection with poliomyelitis. Even routine passages of the less virulent strains, were almost impossible to carry through. Under these conditions it was found necessary to discontinue the mouse experiments during the months of August through October.

On account of the international situation the supply of monkeys was very limited. Infectiosity tests could not, therefore, be carried out to the desirable extent, and titrations were out of question. Before being used the animals were subjected to a tuberculin test and their rectal temperature recorded during at least one week.

CHAPTER III.

Measurement of virus activity.

Many of the properties of viruses can be studied only indirectly by determining the effect of various treatments on the infectivity. Thus, it is obvious that advances in the knowledge of viruses is largely dependent upon the accuracy of the methods with which the activity is to be measured. It was, therefore, deemed necessary to pay special attention to questions connected with this problem.

Review of methods.

When the content of living bacteria of a given material is to be estimated, either one of two methods can be used, the plate count or the dilution method. There are advantages and disadvantages connected with both methods. The interpretation and evaluation of experimental data has been subjected to statistical analysis by several authors (56, 58, 93, 102, 124, 145).

In determining the activity of a given virus material one must make a distinction between the number of virus particles and the virulence of the strain of virus. Unfortunately this has not always been the case. From some experiments in the literature it is impossible to decide, whether an observed "attenuation" of a virus is due to a decrease in titer or to be ascribed to a real change in the virus itself. In this chapter only the enumeration of active virus particles is considered.

By analogy with the bacterial count two different procedures for particle count of viruses have been developed. The "local lesion" titration corresponds to the plate count method. This procedure was originally introduced into plant virology by Holmes (64), who found that certain species of tobacco plants produced discrete lesions in-

stead of systemic infection, when their leaves were rubbed with mosaic virus containing plant juice. The number of lesions was dependent upon the virus content of the sap. Holmes compared the local lesions with bacterial colonies on an agar plate. The lesions no doubt represent virus colonies, but as a method of particle count Holmes' procedure differs fundamentally from the plate count. The surface of a tobacco leaf has only a limited number of entry points for the virus, mainly hair cells, which endure the mechanical injury necessary for letting the virus enter, and yet survive, thus permitting the multiplication of the virus. On the other hand each entry point can give rise to only one lesion regardless of the number of virus particles that actually enter. On the basis of these assumptions Youden, Beale and Guthrie (166) have derived a formula for the relationship between number of lesions and actual virus content of the inoculum. This formula is

$$y = N(1 - e^{-ax})$$

where y is the probable number of lesions, N the maximum number of lesions, i. e. the total number of entry points, x the virus concentration, a a constant and e the base of natural logarithms.

It is thus evident that Holmes' method does not permit the evaluation of the actual number of virus particles present in a given solution. The titer value is only relative and must be compared with a standard. But as a method of evaluating the relative virus content it has been developed to great accuracy by Samuel and Bald (134) and by Youden and Beale (165).

In animal virology a similar method has been based on the discovery by Woodruff and Goodpasture (161) that the fowl-pox virus could grow on the chorioallantoic membrane of the developing chick, thereby producing easily recognizable proliferative discrete lesions. Since 1931, when this paper was published, a large number of viruses have been found capable of propagating on the chorioallantois and the technique has been used by Burnet, Keogh and Lush (22) for titration purposes, especially in connection with immunological studies. When proper precautions are taken the method seems to be very sensitive and yield results of great accuracy. So far no statistical analysis of the membrane technique has been published, but it must be considered probable that the analogy to the plate count is more strict than in the case of Holmes' method. From a

theoretical point of view it is probably identical with the plaque count of bacteriophage.

In special cases the local lesion method has been applied to other substrates than the chick membrane, for instance with herpes and vaccinia viruses on the rabbit cornea.

The applicability of the methods now mentioned is, however, limited to a comparatively small number of viruses. In most cases one has to rely on the dilution method. The technical details are in all essential features identical with those in bacterial count. The test material is serially diluted and from each dilution a series of inoculations is made on the test substrate, in this case experimental animals or plants. If the dilution series is extended far enough, there will be some groups of test inoculations showing less than 100 per cent takes. The result of the titration can be expressed in terms of the 100 p. c., the 50 p. c., or the zero endpoint. Of these values only the 50 p. c. endpoint can be estimated with any degree of accuracy. Accordingly a minimal infective dose (MID) is given as the amount of virus that produces symptoms in 50 p. c. of a series of inoculated test animals. The definition of a minimal lethal dose (MLD) is analogous. As in the case of Holmes' method the titer value is only relative and for the same reasons. The number of entry points is limited to certain susceptible cells in a mass of resistant tissue.

As first used in virus research the dilution method resembled the rough gas titer in water bacteriology. From each one of a series of tenfold dilutions one animal was inoculated. The titer was given as the maximum dilution that produced symptoms. It was not uncommon that "skips" occurred in the series of results. Doerr (30) pointed out that the titer value could be rendered more accurate and "skips" be avoided, if several animals were inoculated from each dilution. The 50 p. c. endpoint titration in its present form was suggested by Parker and Rivers (115). Usually each test group consists of at least five animals. The result is expressed in terms of MID or MLD per unit of volume.

The 50 p. c. point is usually calculated from the experimental data through interpolation between two successive frequency figures. Thus in calculating the result one uses only two out of five or six groups of animals, which from a statistical point of view is a waste of material. Therefore, Reed and Muench (125) have suggested that the calculation of the titer value be made not from the actual fre-

quency figures but from their accumulated sums. The result of this operation is a weighted mean and thus more reliable.

The accuracy of the 50 p. c. endpoint titration method has been tested by Horsfall (66) on influenza virus. Using Reed and Muench's modification he found a mean deviation of $\pm$ 0.17 expressed in terms of log dilution, when the test groups comprised five animals.

It is obvious that statistical methods and formulas, developed for the purpose of evaluation of bacterial counts, are applicable to any suspension of particulate material in a fluid and thus must hold true also for virus suspensions. The formula

$$P = 1 - e^{-ax} \qquad (1)$$

for calculation of the probability of takes, P, at any given concentration of virus, x, when amounts of the volume a are being inoculated, has been experimentally tested by Parker with vaccinia virus (112). This author found a fair agreement between the actual rate of takes and the calculated values. We will return later to Parker's experiments.

In most cases of experimental virus infections the incubation period, i.e. the time interval between inoculation and appearance of symptoms, is an almost constant quantity. Sometimes, however, the length of the incubation period is largely dependent upon the amount of virus inoculated. It seems logical to attempt to use this additional information about the quantity of virus inoculated for titration purposes. Bryan and Beard (17, 18) found a linear relationship between log dilution and incubation period in virus induced rabbit papilloma. This relationship can be expressed in the following formula

$$t_1 - t_2 = b'(\log D_1 - \log D_2) \qquad (2)$$

where t_1 and t_2 are incubation periods corresponding to two different dilutions of the same material with the dilution factors D_1 and D_2 resp. They found that the slope of this line measured by the factor b was constant regardless of the origin of the virus or the resistance of the rabbits. Comparing the titration curves obtained with the same virus preparation but in animals of different susceptibility, they found that the two straight lines ran parallel but at a certain distance from each other. These observations led to the following titration procedure. In a series of experiments a standard curve was obtained.

from which the factor b could be calculated. In an experiment with purified virus this standard curve was fitted to the 50 p. c. endpoint titer and both correlated to the protein content of the preparation. In an actual titration the test material is inoculated into two or more animals, which at the same time receive a known amount of a standard virus. The incubation times are read off and the mean difference between test and standard calculated. From the standard curve the corresponding difference in activity can be computed and the result expressed in terms of MID or mg virus protein per unit volume.

The present writer (47) has described a similar procedure for titration of certain strains of mouse poliomyelitis virus. In this case, however, there exists a linear relationship between the reciprocals of the incubation periods and log dilution. Thus, the incubation time formula is

$$1/t_1 - 1/t_2 = b\,(\log D_2 - \log D_1) \qquad (3)$$

The factor b is not constant but varies with the resistance of the animals. Construction of a standard curve was, therefore, not feasible. There exists, however, a definite relationship between incubation time and 50 p. c. endpoint titer, that can be expressed as follows:

$$\log a = 1/bt - c \qquad (4)$$

when a is the number of MID inoculated, b the above mentioned factor, t the incubation time. From several experiments with different virus material c was determined to approximately 1. A titration is done as follows. Suppose there are several tests to be compared with a standard. From the standard a series of five tenfold dilutions is prepared, from each test two different dilutions. With each dilution a group of five or ten mice is inoculated intracerebrally with a standard amount (0.02—0.03 ml.) In each group the harmonic mean of the individual incubation periods is calculated. On the basis of the data obtained with the standard material a standard curve is constructed. Through interpolation in this curve the relative titers of the tests can be evaluated.

About the two last-mentioned methods the following remarks may be made. The appearance of symptoms seems to coincide with the maximum concentration of virus in the infected tissue. This

critical concentration is remarkably constant for a given virus and a given host. Probably only a limited number of virus particles can be produced in each susceptible cell. When this stage has been reached the cell ceases to function normally or disintegrates, and when the number of injured cells has extended sufficiently, clinically manifest disturbances appear. The regularity of the dependence of the incubation period upon the dose of inoculum, as demonstrated in the formulas (*2*) and (*3*), makes the assumption probable that the rate of multiplication of virus in the infected tissue, too, is a phenomenon of great regularity that could be expressed mathematically. Let us assume that one single virus particle for the multiplication to the critical concentration needs the time t. Let further y be the virus concentration at the time x after inoculation. Then $(t - x)$ should represent the incubation time in the case of y particles being inoculated. In the case (*2*) we have

$$t - (t - x) = b\,(\log y - \log 1) \qquad (5\,a)$$

as log concentration corresponds to -log dilution. This leads to

$$y = e^{mx} \qquad (5)$$

where m is a constant. This is exactly the same function that is valid for a growing bacterial population in the logarithmic phase, and it means that each virus particle at constant intervals gives rise to a fixed number of new particles. To the writer's knowledge no attempts have been made to verify this formula by direct observations.

In the case of mouse poliomyelitis we have

$$1/(t - x) - 1/t = b\,(\log y - \log 1) \qquad (6\,a)$$

or

$$y = e^{mx/(t-x)}. \qquad (6)$$

In this formula the exponent contains the variable factor $m/(t-x)$, which increases with growing x. The rate of growth, therefore, should be "overlogarithmic" and the process of multiplication represent an autocatalytic phenomenon.

It is difficult to visualize this autocatalytic process. Probably the generation time of new particles is decreasing with growing

concentration of virus, perhaps on account of a gradual disappearance of some inhibiting influence. The latter assumption might explain the difference in the influence of resistance on the factor *b* in cases (*2*) and (*3*). Resistance to papilloma infection causes only a parallel displacement of the titration curve, which means that either the number of entry points, i.e. susceptible cells, is smaller, or else a certain number of virus particles are destroyed before having an opportunity to come in contact with their substrate. Once in the cells they are no longer subjected to inhibitory influence and the rate of growth remains unaffected. The resistance to mouse poliomyelitis on the other hand should partially consist of an increase of the normally existing tendency to inhibition of the rate of growth. Hence the effect on the shape of the titration curve.

The membrane method.

As the chorioallantois technique seems to be simple as well as accurate, it was decided to test this possibility in spite of the fact that several attempts by various authors to propagate human as well as mouse poliomyelitis virus in the developing chick have given entirely negative results. /Burnet (20), Gavrilov and Fester (51), Theiler (pers. comm.)/.

By inoculation of the eggs the following simple technique was employed. After surface disinfection with alcohol the shell was punctured with a needle, the point of which was blunted so as not to injure the membranes. Two punctures were made, one over the air sac, the other over the embryo. Through suction the air sac was emptied and a new artificial air space produced over the embryo. In the transilluminator it was controlled that the chorioallantois had parted from the shell. The fine needle-point of a tuberculin syringe was inserted through the puncture into the artificial air sac and the material inoculated, the dose of inoculum being 0.05 ml. The openings in the shell were sealed with paraffin and the egg returned to the incubator. The eggs were incubated at 37° C before as well as after inoculation.

Experiment 1. In the first series of experiments the eggs were inoculated after 10—12 days' incubation, i.e. at an age when the specific lesions of the chorioallantois are most easily produced (21). Series of ten eggs were inoculated

with a bacteriologically sterile 10 p. c. suspension of infected mouse brain in physiological saline. At different intervals after inoculation two eggs were opened and the membranes and embryos examined. Lesions of the unspecific type were frequently found on the membranes from one to four days after inoculation. No specific proliferations were observed and no gross lesions in the embryo. Sections were made and examined microscopically. No changes of the specific type were found. Part of the membranes and embryos were ground with sterile sand and saline and inoculated intracerebrally into mice. All test animals were observed during four weeks without showing any symptoms and when tested for immunity proved to be not immune. A few eggs were allowed to hatch in due time. The chickens were quite normal. The results were so far completely negative.

Since, however, Kligler and Bernkopf (83) were able to propagate rabies virus in the chick embryo by using very young embryos for inoculation this technique was tested as well.

Experiment 2. Using the FA strain of mouse poliomyelitis virus the following experiment was set up. A series of ten eggs was inoculated by twos at the age of 4, 5, 6, 7, and 8 days. The embryos in the first pair both died on the day after inoculation, the remainder were all alive at the termination of the experiment. Of each pair one egg was opened on the fifth, the other on the tenth day after inoculation. Membranes and embryos were examined as usual. No gross lesions were observed. The embryos were decapitated and the vertebral column removed. Membranes, CNS, and corpus of the embryo were ground separately with sand and saline and inoculated intracerebrally into groups of five mice. The animals were observed during four weeks and surviving mice tested for immunity. The result of the activity tests is summarized in table II.

The result is very clearcut. In no instance virus could be discovered in the eggs five days after inoculation whereas ten days after inoculation the CNS was infective in embryos inoculated at the age of five, six and seven days but not in eggs inoculated at eight days of age. In no instance virus was discovered in membranes nor in the corpus minus the CNS.

In three more experiments of this kind essentially the same results were obtained. Inoculation at the age of 4 days generally caused premature death of the embryo, whereas attempts to infect 7 days old eggs in two instances were without success while in two instances comparatively small quantities of virus were present in the CNS.

Experiment 3. A passage experiment was performed as follows. Infective CNS from an embryo inoculated at the age of 5 days in one of the above experiments was passed to three eggs 6 days old. On the 10th day after inocula-

Table II.

Activity tests on eggs inoculated with the FA strain.

Age of egg in days at time of inoculation	Test made at time in days after inoculation	Material tested	No. of mice inoculated	No. showing symptoms	No. immune survivors
5	5	membrane	5	0	0
		CNS	5	0	0
		corpus	5	0	0
5	10	membrane	5	0	0
		CNS	5	5	—
		corpus	5	0	0
6	5	membrane	5	0	0
		CNS	5	0	0
		corpus	5	0	0
6	10	membrane	5	0	0
		CNS	5	5	—
		corpus	5	0	0
7	5	membrane	5	0	0
		CNS	5	0	0
		corpus	5	0	0
7	10	membrane	5	0	0
		CNS	5	2	0
		corpus	5	0	0
8	5	membrane	5	0	0
		CNS	5	0	0
		corpus	5	0	0
8	10	membrane	5	0	0
		CNS	5	0	0
		corpus	5	0	0

tion the eggs were opened, the CNS harvested, pooled, ground with sand and saline, and passed to a new set of three 6 days old eggs. At the same time a titration in mice was made. One more passage of the same kind was made and the last harvest inoculated into 15 eggs 10 and 11 days old. 10 days after inoculation the CNS was collected in the usual way and a pool of the material tested for activity in mice. The results of the titrations are summarized in table III.

The activity was thus maintained unchanged through four passages but the virus was still not infectious to older eggs.

Table III.

Activity tests on egg passages of the FA strain.
Test made 10 days after inoculation.

Passage no.	Age of eggs at time of inoculation	Virus content of CNS (log MID per gm.)
2	6 days	4.15
3	6 »	4.80
4	6 »	4.15
5	10—11 »	no virus

Numerous sections of brain and spinal cord from infected embryos were examined microscopically. A few slides suggested a slight increase in the number of cells but nowhere any definite and characteristic lesions could be detected.

One more strain of mouse virus has been transferred to eggs. This strain, designated as UF I, of fecal origin, was of moderate virulence, the attack rate being about 80 p. c., the incubation time 10—15 days and the symptoms exclusively spinal. 6 days old eggs were inoculated and harvested, part after 5, the remainder after 10 days. No virus could be detected in the 5 days' harvest but two mice out of ten inoculated with CNS from the 10 days' harvest came down with typical symptoms on the 15th and 17th day.

Attempts were also made to transfer human poliomyelitis virus to eggs. For this purpose the L strain was employed, originally isolated by Levaditi in 1911 and since then maintained in monkey passages. Unfortunately these experiments had to be interrupted before any conclusive results were obtained. Even at the start of the experiments described above certain difficulties were encountered in procuring eggs of a satisfactory quality. In the winter 1941—42 when the attempt now mentioned was made, the scarcity of eggs was pronounced and obtainable hatching eggs of low quality, probably on account of vitamine deficiency of the chicken food. Most eggs, inoculated or not, died on the fifth to tenth day of incubation and it was practically impossible to carry through any passages even if as much as twenty eggs were used in each series. Under these circumstances the experiments were interrupted for the time being.

The experiments now described leave no doubt that the mouse poliomyelitis can be transferred to and propagated serially in the

developing chick, if indefinitely has not been settled as yet. Though this fact might be of a considerable theoretical interest, no practical application could be made. The immediate purpose of the experiments was to find a method for titration of the virus. As no characteristic lesions are produced, the diagnosis can be settled only indirectly through passage to mice, and transfer to eggs is therefore at present of no use either for titration or diagnostic purposes. As the virus concentration in the infected embryo remains very moderate, it offers no advantages as source of virus either.

The incubation time method.

The main principles of this procedure have already been outlined by the present writer. As, however, accuracy in the measurement of biological activity is of the utmost importance for the solution of many problems of physico-chemical nature, a thorough examination of the method from this point of view was necessary. A survey of this kind must be based on a purely statistical reasoning, as individual variation is one of the most prominent characteristics of a biological material. In the choice of statistical methods the presentation by Bonnier and Tedin (15) of the theories of Fisher and others has mainly been followed.

In a routine titration a number of tests are compared with a standard. It is essential that all samples be derived from the same strain of virus and preferably of the same origin, the tests being different fractions or samples of the standard treated in different ways. The technique employed was in detail as follows.

From the standard a series of five tenfold dilutions was prepared, usually with log dilution 2, 3, 4, 5, and 6. From each test sample two dilutions were prepared: 0 and 2, 2 and 4, or 4 and 6, depending upon the range of activity to be expected. In diluting 1: 10 1 ml was pipetted into 9 ml of diluting fluid, 1: 100 0.25 ml into 25 ml. All pipet readings were made between marks and clean pipets used for each dilution. 0.05 m (= 0.3 p. c.) sodium chloride solution was generally used for dilution. The inoculations were made with tuberculin syringes with glass pistons and needles gauge 20. The syringes were sterilized by boiling in water and before filling washed twice with the test sample in question. 30 days old mice served as test animals, the difference in age not exceeding 3 days. Uniformity of weight was usually not considered. From each dilution 0.02 ml amounts were inoculated intracerebrally into 10 mice.

With one assistant anesthetizing the animals it was possible to inoculate 400 mice per hour. The animals were kept by fives in glass jars, observed once or twice daily through at least three weeks, and their symptoms noted. When characteristic symptoms had appeared, the animals were sacrificed and the brains collected for purification purposes. After termination of the experiment all surviving animals were tested for immunity with a large intracerebral dose of the virulent FA strain.

In table IV the result of a typical titration experiment is tabulated. Each row represents a group of test animals inoculated with the same amount of the same dilution of a test material. The columns contain the numbers of animals, showing the first symptoms of infection on the day after inoculation which is indicated in the head of the column. The last one of these columns contains animals not becoming sick, the incubation period in these cases being indefinite.

Table IV.

Result of a typical titration experiment.

Test material	Log dilution	Number inoculated	Number becoming sick after days											S (y)
			2	3	4	5	6	7	8	9	10	11	∞	
12	2	10	1	6	3									3.25
	4	9			3	4	1	1						1.860
13	2	9		6	3									2.75
	4	10				9		1						1.943
14	2	10		2	7	1								2.617
	4	10				7			1	1			1	1.636
15	2	10		4	5	1								2.783
	4	10				6	2	1	1					1.801
16	2	10		6	3									2.75
	3	10			4	3	1	2						2.052
	4	10				6	1	3						1.795
	5	10					3	1	4	1			1	1.254
	6	10								1	1	1	7	0.302
17	2	10		2	7		1							2.583
	4	10			1	2		5	1				1	1.489

From a statistical point of view the experimental data form a problem of covariation and should be analysed accordingly. Independent x-variable is log dilution and dependent y-variable the re-

ciprocal of the incubation period. In the last column of the table the group totals of the y-values are listed.

To each test there corresponds a line of regression given by the equation

$$Y = \bar{y} + b(x - \bar{x}) \tag{7}$$

$\bar{y}$ and $\bar{x}$ being the means of y and x and b the coefficient of regression, always negative when x denotes log dilution but positive when x denotes log concentration. This considered, equation (7) is identical with the incubation time formula (3) on page 35.

For each test, therefore, a coefficient of regression, b, can be calculated. The titer values obtained from the titration data are compatible only on the condition that the test groups show conformity with regard to regression of y on x. In a number of experiments the variation of b was studied from this point of view. The result, expressed as usual in terms of the probability of the differences observed being a chance variation, was as follows:

Probability (P)	No. of experiments	
0.001—0.01	1	
0.01—0.05	5	
0.05—0.2	7	32
0.2—1.0	25	
	Total 38	

In 32 out of 38 experiments the variation was probably due to chance. In 5 experiments a significance of the 1st order was observed, and in one a significance of the 2nd order. The result suggests largely a normal chance variation.

Provided the cause of variation in b, if not due to chance, be detected and eliminated, the titer values in an experiment like that in table IV can be calculated as follows. Let Y_0 correspond to the dilution $x = 0$. According to equation (7) we have

$$Y_0 = \bar{y} - b\bar{x}. \tag{8}$$

Y_0 is a measure of the activity of the undiluted material. In order to obtain an expression of the usual form, i.e. in terms of log dilution we must divide through $-b$. Letting A be the activity, we get

$$\log A = \frac{\bar{y}}{-b} + \bar{x}. \tag{9}$$

Letting the index t denote a test sample and s the standard, we have

$$\log \frac{A_t}{A_s} = \frac{\bar{y}_t - \bar{y}_s}{-b} + (\bar{x}_t - \bar{x}_s). \qquad (10)$$

The relation between A and the MID titer is expressed in the formula (4) on page 35. Considering that the constant c in this formula is approximately 1.0 and that the volume of the inoculum is 0.02 ml, the virus content might be estimated to approximately 5. A MID per ml of the undiluted solution. This relationship will, however, be studied in detail later.

According to formula (9) the accuracy of the titer value is dependent upon the variation of x, y and b. The former variation is principally connected with the accuracy of pipetting and can not be dealt with in this connection. The variation of y can be divided into two constituents, one of which refers to the regression of y on x, the other involves differences in the individual resistance to infection, errors of administration and distribution of the material inoculated etc.

Variation of regression.

a) *Course of the line of regression.* In the analysis made above it was implied that the line of regression had a straight course. As already pointed out there is no easily conceivable theoretical basis for this assumption. As an entirely empirical observation its validity had to be statistically verified.

Suppose that the line of regression is curved. On that condition b in the formula (7) should be a variable, dependent upon y. This possibility can be analysed statistically by calculation of the regression of b on y. This was done in 26 different titration experiments. In the individual experiments the coefficient of regression (b on y) was found to vary from -0.502 to $+0.728$ with a mean value of $+0.035$. With a P value of 1.0 it was not significantly different from 0. The coefficient of correlation was calculated to $+0.105$, this neither significantly different from 0, the P value being 0.2—1.0.

Furthermore in a series of experiments the deviation from a rectilinear regression was analysed in the usual way, and the probability of the deviation being due to chance was calculated. The result was as follows:

Probability (P)	No. of experiments	
< 0.001	2	
0.001—0.01	1	
0.01—0.05	3	
0.05—0.2	8	22
0.2—1.0	14	
	Total 28	

Thus, in 22 out of 28 experiments the deviation observed was insignificant, in 3 significant of the 1st order, in 1 significant of the 2nd order and in 2 significant of the 3rd order. This distribution suggests a normal chance variation, except in two experiments, where the difference observed was probably due to technical inaccuracies.

There is, therefore, no evidence to indicate that the line of regression should deviate from a straight course in the interval used by titration. For extremely small or extremely great values of y one must expect a definite deviation. We will return later to this question.

b) *Technical errors* could certainly cause a variation of b. The amounts of solution inoculated are too small to be accurately measured with the syringes used, particularly if one considers the large series of animals to be inoculated in a single experiment.

Experiment 4. In a few experiments the influence on y and b of a variation in the volume of the inoculum was studied. Standard amounts of 0.01, 0.02, and 0.03 ml of the same virus solution, measured as accurately as possible, were injected intracerebrally to groups of 30 days old mice. The incubation periods were noted as usual. The results of three such experiments are summarized in table V.

The relative titers were calculated on the basis of a mean value of b derived from a very large series of experiments. In each case the virus content of the 0.01 ml dose was taken as standard, and the expected values of 0.02 and 0.03 ml calculated on this basis.

Table V shows considerable differences between found and expected. All those differences are positive. Furthermore their amount seems to be proportionate to y. This is illustrated in fig. 1, where the 0.02 ml values are fitted to the normal line of regression. The y values from the 0.03 and 0.01 ml groups were expected to fall on the two parallel dotted lines. The actual position of the points

Table V.

Influence on the incubation period of variation in the volume of the inoculum.

Experiment	Number inoculated	Volume inoculated	$\bar{y}$	Titer values	
				observed	expected
a	49	0.01	0.188	0.00	
	50	0.02	0.231	0.71	0.30
b	49	0.01	0.170	0.00	
	50	0.02	0.195	0.42	0.30
	50	0.03	0.222	0.87	0.48
c	50	0.01	0.292	0.00	
	48	0.02	0.351	0.98	0.30
	50	0.03	0.392	1.66	0.48

suggests that the lines of regression have a different slope, i.e. the value of b is variable, dependent upon the volume of the inoculum. The significance of this observation will be discussed elsewhere. In the present connection it is sufficient to remark that the variation in incubation period resulting from technical inaccuracy of administration is of a higher order of magnitude than the causative irregularities of dosage.

The best way to minimize the "personal" error in administration of the inoculum would probably be the use of spring steered stop syringes, automatically delivering a fixed amount of solution. On account of the existing circumstances it proved impossible to obtain instruments of that kind. The tuberculin syringes used instead were far from ideal for the purpose. They showed a marked tendency to leakage on the side of the piston, particularly when injection was applied against pressure, as is the case by intracerebral inoculations. The leakage was most pronounced when the piston was in topmost position and decreased when the piston was moved towards the bottom of the syringe. In order to standardize this error it was decided to fill the syringes only partially never exceeding a certain limit. Furthermore air bubbles must be removed. If not they would serve as air cushions and cause uncontrollable variation of dosage, when injection against pressure was applied. Finally, it was always

controlled that the total amount of material administered to one test group of animals was correct. This was done in order to make the total plus and minus errors as close to zero as possible.

One more source of error to be considered in this connection is the leakage from the site of inoculation through the needle puncture almost invariably occurring when the syringe is withdrawn. In a series of experiments on dead mice this leakage was found to be

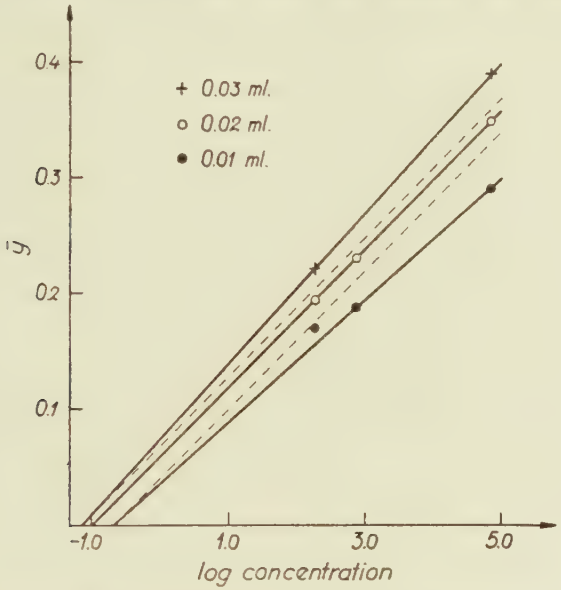

Figure 1. The influence of the volume of the inoculum on the length of the incubation period and the slope of the titration curve. Experiment 4.

astonishingly constant, amounting to about 0.002 ml. regardless of the quantity inoculated. In all calculations regarding absolute quantities, therefore, this correction should be made.

c) *The animal error.* In a series of experiments the individual resistance against infection and its dependence upon different factors were studied.

In all titration experiments each test group of ten animals comprised five males and five females. Calculations of the titer were done for both sexes separately. As the results regarding the relative resistance were invariably the same, it might be sufficient to quote one such experiment.

Table VI.

Experiment 5. Comparison between the sexes.

Test	x	Number becoming sick after days															$S(y)$
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	∞	
a. Males, groups of 5 mice.																	
8	2	1	1	2	1												1.15
	4			1	1	1	1	1									0.746
10	2	4	1														1.583
	4		1		3	1											0.893
11	2	1	1	3													1.183
	4				2	1	1									1	0.601
12	2	5															1.667
	4		2	1	2												1.033
21	2	4	1														1.583
	3	1	2	1	1												1.2
	4		1	3	1												1.017
	5				2	1							1			1	0.548
	6					1		1	1	1						1	0.445
b. Females, groups of 5 mice.																	
8	2	4		1													1.533
	4			2	1	1										1	0.710
10	2	5															1.667
	4			1	4												0.867
11	2		5														1.25
	4				2	1			2								0.676
12	2	4	1														1.583
	4		2	3													1.1
21	2	4	1														1.583
	3		2	1	1		1										0.992
	4		1		3		1										0.875
	5				3			1							1		0.674
	6					1	1	2								1	0.490

Experiment 5. Four unknown fractions from a centrifugation experiment were compared with the untreated standard. All technical details were in conformity with the general description on page 15. The result is summarized in table VI.

An analysis of the result showed with a probability P of more than 0.2 that the sexes behaved as random samples of the same statistical population. In particular the coefficients of regression, —0.05406 and —0.05652, were identical with a probability of more than 0.2.

The influence of weight upon the resistance was analysed in the following experiments.

Experiment 6. A number of mice 31 to 34 days old were weighed in the morning before feeding and separated into groups according to weight, the mean weights of the groups being 9, 11, 13, and 17 g. Each mouse was inoculated intracerebrally with the standard amount of the same virus solution. The animals were observed as usual and the incubation periods noted.

Table VII.

Weight and resistance.

Sex	Mean weight	Number inoculated	Number becoming sick after days					$\bar{y}$
			3	4	5	6	7	
			a.					
♂	9	12	1	5	3	3		0.2236
	11	21	3	7	9	1	1	0.2314
	13	23	7	9	5	2		0.2573
	17	5	3	1	1			0.2900
♀	9	23	7	11	4	1		6.2630
	11	29	7	15	5	2		0.2557
	13	15	4	7	3	1		0.2567
			b.					
♂	9	13	3	6	2	1	1	0.2469
	11	21	3	12	5	1		0.2460
	13	10	3	3	4			0.2550
♀	7	11	3	2	4	2		0.2394
	9	31	11	14	6			0.2699
	11	10	2	2	6			0.2367
	13	8	3	4	1			0.2750

In an identical experiment four groups of mice were inoculated. The mean weights were 7, 9, 11, and 13 g.

The results of these two experiments are summarized in tables VII a and b.

If these two experiments are analysed together one will find a regression of y on weight of $+0.004$ and a correlation of $+0.130$, which with a probability of slightly more than 95 p. c. is different from zero. It is, thus, probable that big animals are less resistant than small animals of the same age. At first sight this fact might seem surprising. It must be kept in mind, though, that the big animals come from numerically small litters, the reduction in number of the young often being due to an abnormal mortality rate, indicating a low general resistance through inheritance. As the regression of y on weight, according to these experiments, was very low, it was not considered necessary to pay special attention to differences in weight among the test animals; the only precaution taken was to avoid letting any test group consist exclusively of unusually big or unusually small mice.

It has previously been pointed out that the age of the test animals has a very pronounced effect on the incubation period. This fact is clearly illustrated in the following experiments.

Experiment 7. Four groups of mice, 4, 6, 8, and 10 weeks old, were inoculated with the standard amount of the same virus solution. The animals were observed as usual and the incubation periods noted. The result is summarized in table VIII.

Table VIII.

Influence upon the incubation period of the age of the test animals.

Age of mice	$\bar{y}$	σ	
4 weeks	0.2897	0.05512	Regression of $\bar{y}$ on age $-$ 0.012
6 »	0.2527	0.05824	correlation $-$ 0.385
8 »	0.2439	0.07427	Regression of σ on age $+$ 0.003
10 »	0.2114	0.07197	correlation $+$ 0.891

The experiment shows that y is dependent upon the age of the mice. The coefficient of correlation is -0.385 and of a very high significance. Furthermore it can be seen from the table that the standard deviation (σ) in a group of animals has a high positive

correlation to the age of the group. The coefficient of correlation +0.891, is, however, not quite significant.

Experiment 8. Infected mouse brains were ground with sand and saline to a 10 p. c. suspension and centrifuged. From the supernatant a series of 5 tenfold dilutions was prepared. From each dilution two groups of test animals were inoculated intracerebrally with the standard amount. One group comprised mice, 43 days old, the other group consisted of animals the age of which varied from 54 to 92 days. The animals were observed as usual and the incubation periods noted. The result of the experiment is summarized in table IX.

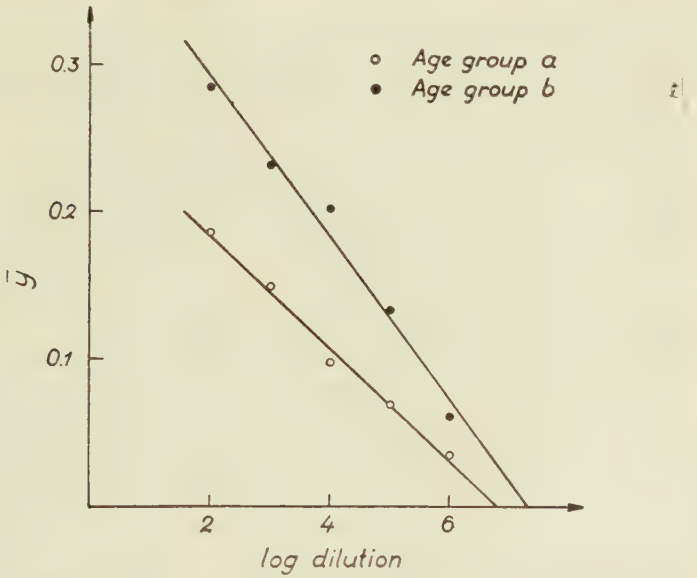

Figure 2. Influence of the age of the test animals on the incubation time curve. Experiment 8.

The experiment showed that not only y but also b is dependent upon the age of the test animals. The coefficients of regression were — 0.03843 and — 0.05559. The probability of this difference being due to chance variation is less than 5 p. c. The experiment is illustrated graphically in fig. 2.

The last experiment demonstrates the limited value of an absolute titer, in terms of MID or otherwise. The titer of a given virus solution is dependent not only upon the virus content but also upon the resistance of the test animals used for titration. Only a comparison between two titer values is of any significance, either as measurement

of virus activity or as an expression for host resistance. When evaluation of the relative virus content is our purpose, it is, therefore, of principal importance to minimize the variation in age of the test animals. The steeper the titration curve the more accurately the titer can be calculated. As furthermore σ increases with age, there is a double reason to prefer young animals for titration purposes. The young are usually not completely weaned until 4 weeks old, which was the reason why this particular age was chosen as standard.

d) *The »reading» error.* The result of a titration depends largely upon the accuracy with which the incubation period can be determined. So far no strictly objective method for registration of the manifestations of disease has been devised. Temperature readings, very valuable in the diagnosis of monkey poliomyelitis, are in this case of no use. One has to rely entirely upon the disturbances directly visible. Often the first sign of the approaching disease is a certain nervousness and hyperirritability. This symptom is, however, very vague and not always present. Furthermore it seems to appear definitely before the maximum concentration of virus is reached. The only reliable symptoms are the characteristic disturbances of motility, in the case of the FA strain mainly convulsions and spastic paralysis. Our aim is to detect the very onset of disease, i.e. the beginning of the second stage. This cannot always be done. After a fit a sort of latency period seems to set in, during which it is impossible to induce a new attack. As the mice otherwise behave quite normally it must, therefore, be a matter of chance whether this early disposition to convulsions will be detected or not. This will be particularly true in cases with a long incubation period, where the disease develops lingeringly and the second stage can extend over four or five days. As a rule, therefore, the incubation periods noted tend to be too long.

The inoculations were usually performed at about 12 noon. The readings were made at 9 a. m. and 6 p. m. The day of inoculation was designated as 0, the following day as day 1, etc. All animals showing the first symptoms at either reading on, say, day 2 were listed for the incubation period of two days and the corresponding y put to 0.5. This involves an approximation. The onset of symptoms for the animal in question had occurred some time between 6 p. m. on day 1 and 6 p. m. on day 2, i.e. in the interval from 1.25 to 2.25 days after inoculation. As it is not feasible to note the exact

Table IX.

Influence of the age of the experimental animals upon the result of titrations.

Age group *a* 54—92 days old, age group *b* 43 days old.

Age group	x	No. inoculated	Number becoming sick after days																				$\bar{y}$	b	log A
			2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	24	∞			
a	2	20		2	6		3	2	3	2	1			1												
	3	28		1	4	2	6	2	4	3	1	1			1				1				2			
	4	32				1	4	5	5	6	1		1	1									8	0.0972	−0.03843	6.79
	5	38						4	4	7	2	3	1		2				1				14			
	6	35							2	2	2	1	1			1	2	1		1	1	1	20			
b	2	8	1	2	3	1	1																			
	3	10		3	3		2		1	1																
	4	10		2	1	2	2		3															0.1849	−0.05559	7.28
	5	4					1	1		2																
	6	10					1		1	2		1											5			

incubation period for each animal an average must be instituted. The arithmetic mean for day 2 should be 1.75. The corresponding value of y can be calculated by integration of the function $y = 1/t$ in the interval 1.25—2.25 and is found to be 0.5878. There is a distinct difference between this figure and the value accepted for calculation of the titer. The difference, amounting to 17.5 p. c. for day 2, rapidly decreases with increasing incubation period, being only 2.65 p. c. for day 10.

The two circumstances now mentioned both act in the same general direction, towards a lowering of the value of y. The difficulty in determining the exact incubation period mainly affects the small y values, the approximated calculation of the average principally the large ones. It is thus possible that the total effect might be a parallel displacement of the titration curve, although it is more probable that the first effect in question does not counterbalance the second. It is therefore reasonable to assume that the relationship between y and log dilution in reality is not linear but represents a curve with the convexity towards the x axis.

As already shown the formula (7) holds true in the interval ordinarily used by titration, with the approximated calculation of incubation periods as originally accepted. From a practical point of view there has been no reason, therefore, to substitute for this procedure a more complicated one even if this might be more correct. From a theoretical point of view, on the other hand, the facts now mentioned are most important and their significance will be discussed in another connection.

In the course of two years a considerable number of titrations were carried out, in which the principles outlined above concerning the uniformity of the test animals were strictly followed. In a series of 34 such experiments comprising more than 4000 test animals the variation in the value of b was studied. The figures obtained suggested a seasonal fluctuation of the general level of resistance. A statistical analysis showed, however, that the differences were within the limits of a chance variation. It might be possible to present conclusive evidence in this matter in the future, when a larger material has been collected. As conditions now were, it was considered most adequate to compute a mean value of b to be applied to all calculations of titer values. This was found to be $b = -0.05473 \pm 0.00077$.

Variation of y.

The variation of y is measured by the mean square residual (σ^2), obtained after elimination of the part of the variation that refers to the regression of y on x. It is obvious that all the sources of error that were reviewed on the preceding pages contribute to this variation. There exists, however, one more cause of variation not yet considered. By random sampling from a suspension of particles in a fluid, the number of particles per sample will show a frequency distribution according to the law of Poisson. This distribution error is a quality inherent in the particulate nature of the virus, and cannot be influenced by any technical device. On certain theoretical conditions to be discussed in the following chapter the amount of this variation can be calculated. For $\bar{y} = b$ it amounts to about 0.0010 and decreases rapidly with growing $\bar{y}$.

The same series of experiments, from which the average value of b was computed, was used for calculation of σ. A mean value of $\sigma^2 = 0.003427$ was obtained. As the above mentioned distribution error contributes but an insignificant part, the variation must be caused by technical inaccuracies. In order to enhance the accuracy of titration there is, therefore, more to be gained by technical improvements than by increasing the number of test animals.

From the value of b and σ the standard error of the titer can be calculated. For 5 groups of 10 mice each one gets $\varepsilon(\log A_s) = \pm 0.158$, and for 2 groups of 10 animals $\varepsilon(\log A_t) = 0.245$, why the difference between test and standard in a routine titration has a standard error $\varepsilon\left(\frac{\log A_t}{\log A_s}\right) = 0.292$.

In the following experiment the actual accuracy of the method was tested.

Experiment 9. Infected mouse brains were ground with sand and saline to make a 10 p. c. suspension, and centrifuged in an angle centrifuge to remove sand and coarse material. The supernatant = solution a. Part of this was diluted with 1/2 volume of saline = solution b. Of each solution a series of tenfold dilutions was prepared and of each dilution the standard amount inoculated intracerebrally to 10 mice of standard age. The animals were observed and readings made as usual. The result is summarized in table X.

According to the above calculations the standard error of each titer value would be 0.158, and that of the difference consequently 0.223. The actual difference in concentration between a and b

Table X.

Accuracy of the titration method.

	x	No. inoculated	Number becoming sick after days																		x	$\bar{y}$	log A
			3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	∞			
	2	9	2	4	3																		
	3	9		2	4	1			1	1													
a	4	8					1	1	1	3	1	1										4.0	0.1377	6.515
	5	9						2	1	1	1	1		2							1		
	6	9					2					1				1				1	4		
	2	10	2	2	6																		
	3	9			4		2	1	1	1		–									2		
b	4	10				1	2	3			2											3.957	0.1259	6.257
	5	7					1		1	1	2	1									1		
	6	10					1	1											2		6		
																						difference	0.258

amounts to $\log A_a - \log A_b = 0.176$. The difference observed in the experiment was 0.258. The error is therefore 0.082, i.e. well within the limits of the standard error.

* * *

Finally it must be pointed out that the incubation time titration method has only a limited field of application. Most strains of mouse poliomyelitis virus, including all intestinal strains, give symptoms exclusively from the spinal cord. When inoculated intracerebrally, they show a practically constant incubation period, regardless of the amount of virus injected. So far only two strains have been isolated, regularly displaying encephalitogenic properties. These strains, designated as GD VII and FA, were both picked up accidentally in passage series of yellow fever virus, and have been described by Theiler and Gard (150). It is only on these strains that the incubation time method of titration can be applied and only after intracerebral inoculation. If the animals are infected by the intranasal or the intraperitoneal route the incubation period will have a constant length regardless of the amount of virus inoculated.

The 50 p. c. endpoint method.

In chapter IV page 66 a large scale experiment on the determination of the 50 p. c. point is reported. In this experiment 15 groups of 50 mice each in the range between 0 and 100 p. c. takes were observed. The standard deviation of percentage of takes from the best fitting distribution curve was calculated to 5.94. When groups of 10 mice instead of 50 are used, the standard deviation would amount to 13.28. If the titration is done with tenfold dilutions an average of 2.7 mouse groups will give a result in the range between 0 and 100 p. c. takes, meaning that 2.7 groups can be used for calculation of the titer value. The standard error of the computed 50 p. c. point will, therefore, be 8.08. The slope of the titration curve in the interval in question, i.e. the increase in percentage of takes per unit of log dilution can be estimated to approximately 40. The standard error of the titer value, expressed in terms of log dilution will, therefore, be $\frac{8.08}{40} = 0.20$.

For titration of an unknown sample according to the 50 p. c. endpoint method usually at least 5 groups of animals must be inoculated in order to ascertain that the favourable interval is covered. For a simultaneous titration of, say, seven different samples 350 mice had to be used. The difference between two titer values could then be estimated with an accuracy of 0.285. With the incubation time method only 170 mice had to be used and the standard error would according to the deductions on page 55 be 0.292, i.e. practically the same accuracy with less than half the number of test animals. For simultaneous titration of a large number of test samples the incubation time method is, therefore, to be preferred.

In a number of suitable experiments the 50 p. c. endpoint titer was calculated together with the log A from the incubation time. In table XI these titer values are compared.

Table XI.

Comparison between 50 p. c. endpoint and incubation time titer.

Incubation time titer	50 p. c. endpoint titer	Difference
6.97	5.75	1.22
4.00	2.88	1.12
8.14	6.25	1.89
7.69	6.58	1.11
7.41	6.25	1.16
6.48	4.58	1.90
7.46	5.75	1.71
6.86	5.25	1.61
7.42	6.00	1.42
7.16	5.67	1.49
6.65	5.67	0.98
5.28	3.77	1.51
5.68	4.11	1.57
6.56	6.38	0.18
6.58	5.88	0.70
6.42	5.30	1.12
7.38	6.39	0.99
7.16	5.86	1.30
7.63	6.36	1.27
	Average	1.277

The constant c in formula (*4*) was previously estimated to approximately 1.0. According to table XI it can be calculated to 1.277 ± 0.067. The individual values are rather widely scattered around the average. As, however, the experiments were not originally planned for calculation of the 50 p. c. endpoint titer, some of these values might be less accurate than the ordinary results of this method. The significance of the difference between the incubation time and the 50 p. c. endpoint titers will be discussed in the following chapter.

CHAPTER IV.

On the theory of titration with reference to certain physico-chemical properties of the virus.

When random samples of a constant quantity are picked from a suspension of particles in a fluid medium the number of particles per sample will show a certain variation around the average. If the distribution of the particles in the medium is due exclusively to chance, i.e. if no attraction or repulsion between particles influences the equilibrium, the frequency series of Poisson can be applied to the problem of calculating the expected number of particles per sample. Let x be the average number per sample. Then in a sufficiently large series of samples the fraction

e^{-x}	will	contain	no particles
$x e^{-x}$	»	»	1 particle
$\frac{x^2}{2!} e^{-x}$	»	»	2 particles
$\frac{x^n}{n!} e^{-x}$	»	»	n »

The probability of a sample containing at least one particle will, therefore, be

$$P = 1 - e^{-x} \qquad (11)$$

Let the particles be bacteria and the samples used as inocula in a series of cultures. The expression (*11*) will then give the expected fraction of positive results.

In the case of a virus titration conditions are partially different. Poisson's law must of course be applicable to the distribution of particles in the suspension, but the percentage of takes cannot be calculated as in (*11*). When the sample is injected into a test animal, the fluid is distributed over a certain amount of tissue. In this

tissue there are a limited number of susceptible cells, serving as entry points. Thus usually only a fraction of the virus particles inoculated have a chance to infect the host. Let the probability of hit be p. If the sample contains n particles, the probability of no hit will be $(1 - p)^n$. The total probability of takes is obtained by addition of the series

$$P = [1 - (1 - p)]\, x\, e^{-x} + [1 - (1 - p)^2]\frac{x^2}{2!}e^{-x} + \cdots$$

$$+ [1 - (1 - p)^n]\frac{x^n}{n!}e^{-x} + \cdots$$

$$P = 1 - e^{-px} \qquad (12)$$

If the functions (*11*) and (*12*) are reproduced graphically with P on the ordinate and log x on the abscissa, one will find that the curves have exactly the same shape, but that (*12*) is displaced in comparison with (*11*) with the amount $-\log p$. From the shape of the distribution curve, therefore, nothing can be learnt about the real values of p and x. Only their product can be calculated.

The virus entity one MID does not tally with a fixed number of virus particles. On the contrary it is variable with p, which in its turn is an expression for the equilibrium between host resistance and pathogenicity of the given strain of virus. Letting v be the virulence of the strain and r the resistance of the host, p can be given the form $p = \frac{v}{r}$. Therefore, p is a measure of the virulence as well as of the resistance. As most of the following deductions are concerned with the latter aspect, p will be called the resistance factor of the probability function.

In the case of vaccinia virus a whole titration can be done in one animal, a procedure guaranteeing the best possible constancy of p. In most instances, however, a comparatively large number of animals must be included. One has, therefore, to expect a certain variation in p, so that the shape of the distribution curve will deviate from that of function (*12*). The actual curve can be regarded as an average of a number of normal curves with different p values, and its shape will be dependent upon the type of variation of resistance. At any rate it must have a flatter course than the normal distribution curve.

As already mentioned Parker (112) tested the validity of function (*12*) on vaccinia virus. His aim was to ascertain whether or not one

single elementary body was sufficient to cause infection. Consequently he studied the distribution curve in the range between 0 and 100 p. c. takes, comparing the experimental data with function (*12*) and with a series of functions easily derived from this one, expressing the probability of takes if 2, 3, etc., virus particles form a minimal infective unit. These functions are

$$P_2 = 1 - (1 + px)\, e^{-px} \qquad (12\,a)$$

$$P_3 = 1 - \left(1 + px + \frac{(px)^2}{2!}\right) e^{-px} \qquad (12\,b)$$

etc.

Each one of these curves has a steeper slope than the normal function, and steeper the higher the order. Parker found good agreement with (*12*), but none with the curves of a higher order. Haldane (57), however, pointed out certain inadequacies in Parker's calculations. When these were corrected the result indicated a systematic deviation from the normal curve. Bryan and Beard (19) suggested that this deviation was caused by variation in resistance among the rabbits, and showed that Parker's figures agree with function (*12*), if a normal distribution of animal resistance was assumed.

Parker, Bronson and Green (113) were able to confirm this in a later series of experiments with different strains of virus. In one of these strains, the culture virus C V I with a very low intracutaneous pathogenicity, however, a marked disparity between experiment and theory was observed. The data were best explained by the assumption that as many as 10 elementary bodies were necessary to cause infection. The authors suggest the explanation that one single focus of infection is insufficient to cause a visible reaction. Not until the density of foci exceeds a certain critical level, the reaction becomes recognizable.

If this interpretation is correct, which seems very plausible, it is, however, questionable whether the formulas (*12a*) etc. should be applied. A comparison between the egg membrane and the intracutaneous titers of the strain C V I indicates that p in this case is of the order of magnitude 10^{-5}. When in a test 0.1 ml is inoculated intracutaneously, the inoculum, therefore, can come in contact with but a very limited number of susceptible cells. It is evident that, with a certain number of hits, the chances that more than one hit

occurs in the same cell are greater, the smaller the number of cells in which the hits are to be distributed. But according to Parker it is the number of foci not the total number of hits that count.

The amount of tissue infiltrated by the inoculum will be called the hit area. Let the number of susceptible cells within this area be a. In the case of n hits the probability, that all occur in the same cell, is $\frac{1}{a^{(n-1)}}$. The probability of 1, 2, 3, etc. hits is $px\,e^{-px}$, $\frac{(px)^2}{2!}e^{-px}$, $\frac{(px)^3}{3!}e^{-px}$, etc. Thus, the total probability of one focus is

$$P_{(1)} = px\,e^{-px} + \frac{1}{a}\frac{(px)^2}{2!}e^{-px} + \frac{1}{a^2}\frac{(px)^3}{3!}e^{-px} + \cdots$$

$$P_{(1)} = a\,(e^{px/a} - 1)\,e^{-px} \qquad (13)$$

On the condition, that 2 foci are necessary to give a visible reaction, the probability of takes will be

$$P_2 = 1 - [1 + a\,(e^{px/a} - 1)]\,e^{-px} \qquad (14)$$

In an analogous way the functions of higher order can be derived.

When the formulas of type (*12a*) etc. are compared with those of type (*14*), one will find that the former represent the limit values of the latter for $a = \infty$. An appreciable deviation does not occur until a has the value of or below 10. The principal difference is a displacement towards greater px values, whereas the slope of the curve is practically unchanged. When only calculation of the slope of the titration curve is concerned, the approximated formulas (*12a*) etc. can be applied, so Parker's figures must be regarded as satisfactory.

In the above reasoning it was assumed that a single elementary body was capable of inciting infection, although the intensity of the process was so low as to make a single focus imperceptible. It might be, however, that the low virulence of the C V I strain is due to a deficiency in some activator necessary for establishment of intracutaneous infection (for instance the soluble polysaccharide antigen). In this case two alternatives are possible.

a. The activator is inherent in the virus particle. Then more than one particle must enter into the same cell to give the amount of activator necessary. Only the case of two particles forming a minimal infective unit will be considered. The symbols are as before. The probability, that n hits all occur in different cells is

$$\frac{a!}{a^n(a-n)!}$$

and, therefore, the probability of at least one double hit $1-\frac{a!}{a^n(a-n)!}$. If n is greater than a it is self-evident that double hits must occur. The total probability of takes is then

$$P=\left[1-\frac{a!}{a^2(a-2)!}\right]\frac{(px)^2}{2!}e^{-px}+\cdots+\left[1-\frac{1}{a^a}\right]\frac{(px)^a}{a!}e^{-px}+$$
$$+\frac{(px)^{(a+1)}}{(a+1)!}e^{-px}+\cdots\cdots$$

By separating the members of the bracket one gets

$$P=\sum_{n=2}^{n=\infty}\left[\frac{(px)^n}{n!}e^{-px}\right]-\sum_{n=2}^{n=a}\left[\binom{a}{n}\left(\frac{px}{a}\right)^n e^{-px}\right]$$

and

$$P=1-\left(1+\frac{px}{a}\right)^a e^{-px}. \qquad (15)$$

b. The activator is mainly present in the fluid medium and does not combine with the virus until *in loco*. Only the simple case that one activator molecule together with one virus particle forms the minimal infective unit will be considered. The total hit area is $\frac{a}{p}$. If one cell is hit by a virus particle, the probability that an activator molecule will hit the same cell is $\frac{p}{a}$, and the probability of no hit consequently $1-\frac{p}{a}$. The probability that of n activator molecules none will hit the cell is $\left(1-\frac{p}{a}\right)^n$. Suppose the solution contains an average of c activator molecules per virus particle. The probability of takes in the case of one hit by a virus particle will then be

$$P_{(1)}=S\left\{\left[1-\left(1-\frac{p}{a}\right)^n\right]\frac{(cx)^n}{n!}e^{-cx}\right\}=1-e^{-cpx/a} \qquad (16\,a)$$

In the same way the probability of takes in the cases of 2, 3, etc. hits by virus particles can be calculated

$$P_{(2)}=1-e^{-2cpx/a}$$
$$P_{(3)}=1-e^{-3cpx/a}$$
etc.

To obtain the total probability of takes one has to multiply each expression (*16 a*) etc. with the corresponding probability of 1, 2, 3, etc. foci[1] and add the

[1] These values can be calculated in analogy with function (*13*), the probability of n hits being distributed over m foci being

$$P_{\binom{n}{m}}=\binom{a-1}{m-1}\frac{m^{n-1}-\binom{m-1}{1}\cdot(m-1)^{n-1}+\binom{m-1}{2}\cdot(m-2)^{n-1}\cdots+(-1)^{n-1}}{a^{n-1}}$$

products. This leads to a rather complicated formula. In order to get an idea as to the form of this formula we will instead of the probability functions mentioned use the approximated expressions of the form $\frac{(px)^n}{n!} e^{-px}$, remembering that the function ensuing is strictly valid only for small values of px and large values of a.

$$P = S\left[(1-e^{-ncpx/a})\frac{(px)^n}{n!}e^{-px}\right] = 1-e^{-px(1-e^{-cpx/a})} \qquad (16)$$

The principal difference between the functions (*14*), (*15*), and (*16*) lies in their dependence on the arbitrary constant, a resp. c/a. (*14*) was practically independent of the value of a; only a slight displacement towards greater px values occurred with decreasing a. (*15*) and (*16*) on the other hand are largely influenced by variations of the constant and the displacement has the opposite direction, i.e. towards greater px values with increasing a.

It is certainly possible to change the value of a within certain limits by using inocula of different volume. A large inoculum will infiltrate a larger amount of tissue, and the number of susceptible cells in the hit area will be greater than when a small volume is inoculated. It would, therefore, be theoretically possible to decide whether a function of type (*14*) or of the other type were the most probable. An experiment to that effect has, in fact, been reported by Sprunt (140, 141). He titrated the virus, using different standard volumes at inoculation, and found that the activity calculated was higher, the smaller the volume of the inoculum. His conclusion was that the effect was dependent upon the concentration of the virus rather than the actual number of virus particles inoculated. This observation is decidedly in favour of one of the functions (*15*) or (*16*). There is, however, one source of error in an experiment of this kind, to which special attention must be paid, before definite conclusions could be drawn. Intact and normally functioning cells seem to be a necessary condition for the multiplication of the virus. When large volumes are inoculated intracutaneously, distortions of the tissue might easily occur, injuring a number of cells so as to make them unsuitable as substrate for the virus. Sprunt's experiment ought to be controlled with due attention to this possiblity.

Observations on mouse poliomyelitis.

With the FA strain of mouse poliomyelitis virus an experiment of the same kind as Parker's was carried out.

Experiment 10 a and b. In a preliminary titration the 50 p. c. endpoint of an ether-treated extract of infected mouse brain was determined. In accordance with the titer value thus obtained a series of 8 twofold dilutions was prepared, calculated to cover the entire range between 0 and 100 p. c. takes. From each dilution standard amounts of 0.02 ml were inoculated into groups of 50 mice of standard age. The animals were observed during 4 weeks and the

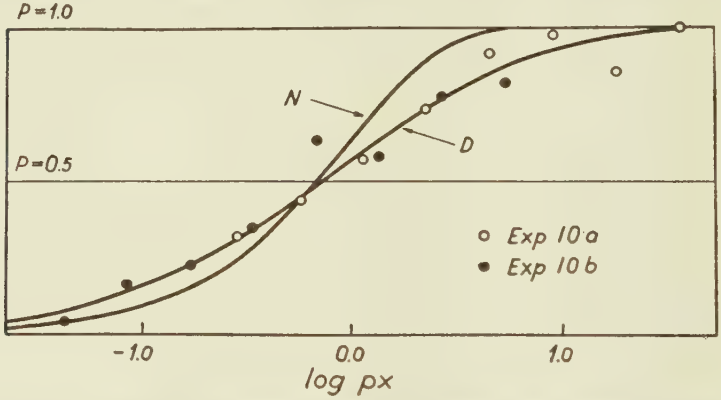

Figure 3. Probability of takes. Curve N = normal distribution. Curve D = distribution in test animals of varying resistance against infection. Experiment 10.

incubation periods noted as usual. The concentrations of virus used turned out to be slightly too high, so an identical experiment was set up with concentrations being 0.15 of those in the first series. In the interval between the experiments the virus solution was stored in the refrigerator, under which conditions the activity remains unchanged for several weeks. The results of the two experiments were very conform, and they will, therefore, be analysed as one single experiment. The result is summarized in table XII.

The 50 p. c. point was calculated according to Reed and Muench, and was found to correspond to the concentration $10^{-6.778}$. In fig. 3 the distribution function (*12*) is reproduced graphically (curve N) and the experimental data plotted so as to let the 50 p. c. point coincide with that of the curve. It is obvious at first sight, that there is a systematical deviation from the expected values. This is confirmed by a statistical analysis. One obtains a χ^2 total of 55.56 corresponding to a probability of identity far less than 0.001. The actual titra-

Table XII.

log dilution	No. inoculated	No. becoming sick after days																		Per cent positive	Per cent single mol.
		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	∞	
Experiment 10 a.																					
5.000	49	10	7	22	7	2		1													100.0	0.0
5.301	48	1	4	14	11	3	4	2	1											8	85.4	0.0
5.602	46		1	10	12	9	5	5	2	1										1	97.8	0.0
5.903	48		2	13	6	7	6	4	3	3										4	91.7	0.0
6.204	49	1		2	8	10	6	7	1		1									13	73.5	0.0
6.505	50			1	7	7	5	4	3		1									22	56.0	0.0
6.806	48				3	7	1	3	4			1	1		1					27	43.7	4.8
7.107	47					2	5	2		3				1	1		1			32	31.9	20.0
Experiment 10 b.																					
5.824	50			6	14	11	4	4			1				1					9	82.0	2.4
6.125	49			2	11	18	3	2			1							1		11	77.6	2.6
6.426	50				12	10	2	2	1								1	1		21	58.0	6.9
6.727	49			1	5	13	1	4	2	2					1	2				18	63.3	9.7
7.028	49				2	3	2	4	1	1					1	1	2			32	34.7	23.5
7.329	49					1	5	1							1	2		1		38	22.4	36.4
7.630	49					1			2	1	1						1	1	1	41	16.3	37.5
7.931	50								1									1		48	4.0	50.0
Total		12	14	71	98	94	49	45	21	11	5	1	1	1	6	5	5	5	1			

tion curve has a course that is decidedly flatter than that of the normal distribution curve. It is, therefore, obvious that the agreement with the different types of curves hitherto discussed must be even less satisfactory, all of these having a steeper slope than the function (*12*). Accordingly there is no reason to suppose that the minimal infective unit contains more than one virus particle.

The percentage of takes as calculated according to the formula (*12*) corresponds to the number of particles per inoculum. The result of experiment 10 might, therefore, indicate that the relative number of particles decreased with increasing concentration, i.e. that the unit particles were associated to larger aggregates. On dilution a process in the opposite direction would occur. By dissociation of virus aggregates two principally different types can be anticipated. (a) Through combination with inhibiting or neutralizing substances inactive aggregates are formed, on dilution liberating active virus particles. (b) There is a tendency to formation of active aggregates or to dissociation into single virus molecules towards an equilibrium dependent upon the concentration of virus.

Both types of reversible dissociation have been observed. The most extensive study in this respect has been made on plant viruses. Mulvania (105) found in 1926 that a partial neutralization of the infectivity of tobacco mosaic virus containing plant juice could be obtained by addition of normal rabbit serum. The infectivity could be restored to a large extent simply through dilution, i.e. a partially neutralized mixture shows a deviation from the normal formula for the distribution of takes. The same phenomenon and even more strikingly is observed at dilution of a mixture of virus and specific antiserum.

Bald (4) found, that crude infective plant juice gave a dilution curve definitely different from the expected one, whereas purified virus showed a high goodness of fit. He stated, however, later (5) that certain pure preparations exhibit the same phenomenon and suggested that it was the result of a dissociation of aggregates. That aggregation can occur has been shown through filtration and stream double refraction measurements by Bawden and Pirie (8) and by Loring, Lauffer and Stanley (100) and the latter authors present evidence to show that this aggregation is reversible by dilution under certain conditions.

From animal virology it is known that the infectivity of partially neutralized viruses can be restored to a certain extent through dilu-

tion, at least in fresh mixtures of virus and serum. The second type of dissociation, however, has not been observed.

Before a discussion of the different possibilites in our case the following remark about the material in table XII should be made. In an experiment of this kind one must expect that a number of animals were infected by a single virus particle, and that furthermore groups of mice could be distinguished infected by 2, 3, etc. particles. According to the formula (7) on page 43 each such group of animals would be characterized by a certain average incubation period. The variation in resistance would, however, scatter the individual values about the average and probably cause an overlapping of successive groups, making them partly indistinguishable. Let t_x signify the incubation period in the case that x particles incited the infection. By inversion of the formula (7) we get

$$\frac{1}{t_{x+1}} - \frac{1}{t_x} = - b \log \frac{x+1}{x}$$

It is easily shown that the difference in incubation period between two consecutive groups will decrease with increasing x. If therefore, any group at all can be distinguished, it should be the one corresponding to infection with a single virus particle, i.e. with the maximum incubation period. From the totals in the last row of table XII it is evident, that one such group is clearly distinguishable in the range from 16 to 21 days, comprising 23 animals. The average $\bar{y}$ was calculated to 0.0545, corresponding to an incubation period of 18.35 days. On the basis of this value the incubation period of the 2 particle group was calculated to 14 days in reasonable agreement with the experimental data.

On the assumption that this interpretation of the experiment is correct, the last column was added to table XII, where the single molecule infections are recorded in percentage of the total number of takes in the corresponding group of animals. After this preliminary remark the discussion of the distribution curve will proceed.

Alternative A. The union of virus and neutralizing substance has been studied extensively by Burnet (22). The »percentage law» (Elford and Andrewes), saying that the proportion of non-neutralized virus particles is independent of the concentration of virus, was explained on the assumption that Langmuir's adsorption isotherm

was applicable to the equilibrium between virus and antibody. It can be formulated

$$k\,c = \frac{100 - P}{P}$$

k being a constant, c the concentration of antibody, and P the percentage of »surviving» virus particles.[1] Let x be the total concentration of virus, x_0 the observed concentration, i.e. the number of active particles per unit volume. In our case $\frac{c}{x}$ will be constant. Burnet's formula can, therefore, be given the form

$$x_0 = \frac{x}{1 + k\,x}. \qquad (17)$$

With increasing x, x_0 will approach an upper limit $\lim x_0 = \frac{1}{k}$, i.e. regardless of the concentration of the material the number of active particles per unit volume will never exceed this value. From the probability function (*12*) it can be calculated that with $px_0 = 5$ the probability of takes is 99.32 p. c., i.e. out of 1000 animals inoculated an average of 7 would remain symptomfree. Among several thousands of mice, inoculated with concentrated solutions of FA virus, no skips have been observed. It is, therefore, evident that in the present case the following inequality must be valid

$$\lim\ px_0 = \frac{p}{k} > 5$$

or

$$k < 0.2\ p$$

p can be estimated to about 0.01 and k, therefore, must have a value below 0.002. In that case, however, the distribution curve obtained through substitution of x_0 for x in function (*12*) will not deviate with an appreciable amount from the normal curve. The percentage law is, therefore, not applicable to the present case.

If on the other hand the law of mass action is applied to the formation of inactive aggregates of virus and inhibitor, one might reason

[1] According to Merrill (103 a) the inactivation is only apparent, the reduction in infectivity instead being due to agglutination of virus particles. In the present connection the interpretation of the phenomenon is of secondary interest as long as the number of active particles, aggregated or not, can be calculated by means of the given formula.

as follows. Suppose that the inactive complex contains m virus particles and n inhibitor molecules. Let x be the total number of virus particles per unit volume and x_0 the number of free particles. The number of inactive complexes will then be $\frac{x - x_0}{m}$. Let further a be the concentration of free inhibitor and cx the total concentration of inhibitor. Then

$$a = c\,x - n\frac{x - x_0}{m}.$$

If k is the dissociation constant, the equilibrium will be expressed through the following formula

$$x_0^m\, a^n = k\frac{x - x_0}{m}. \qquad (17\,a)$$

If c is large, i.e. inhibitor is present in excess, x_0 will increase with x for very small x values, pass through a maximum, and then decrease. The distribution curve will in fact be beautifully fitted by the figures given by Bawden (7). His table 8 (p. 120) is quoted.

Table XIII.

(From Bawden: Plant Viruses.)

Effect of dilution on the infectivity of mixtures of tobacco mosaic virus and sera.

Dilution	Average number of lesions per leaf		
	Saline control	Normal serum	Tobacco mosaic virus antiserum
1/1	240	24	2
1/5	210	40	4
1/25	52	38	9
1/125	29	18	9
1/625	13	5	6

It is obvious, that a distribution curve of this type is incompatible with the figures in table XII. If, however, c is approximately 1 it is possible to find a form of function (*17 a*), which can be fitted to the experimental data. This will be the case if the inactive complex consists of one virus particle and one inhibitor molecule. The following dissociation formula is obtained.

$$x_0 = \frac{k}{2}\left(\sqrt{1+\frac{4x}{k}}-1\right). \qquad (18)$$

The expected frequencies of takes can be calculated according to the formula (*12*) if x_0 is substituted for x. As p in that formula is not known, the absolute value of the dissociation constant cannot be calculated from the best fitting function of type (*18*). We have

$$px_0 = \frac{pk}{2}\left(\sqrt{1+\frac{4px}{pk}}-1\right). \qquad (18\,a)$$

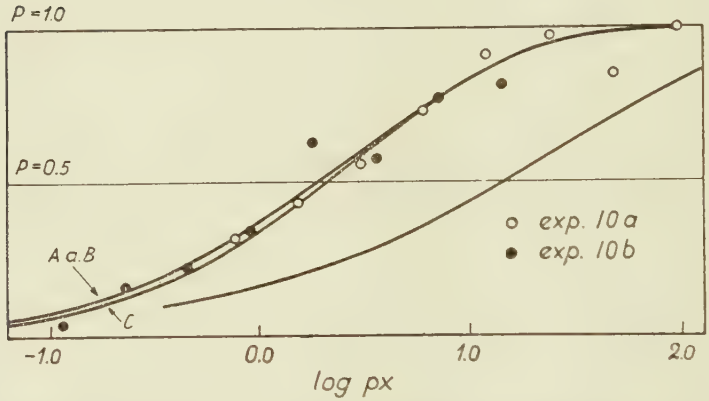

Figure 4. Probability of takes. Curves A—C = distribution according to dissociation formulas A—C. Curve to the right = representation of the function $P = 1 - e^{-K\cdot\sqrt{x}}$. Experiment 10.

It is therefore, possible to find only a value of pk that fits the experiment. This was found to be $pk = 0.4$. The corresponding distribution curve is found in fig. 4 (curve A) together with the experimental data. The agreement is good. χ^2 total was calculated to 15.27, corresponding to a probability of identity of 0.2—0.3. As all active particles in this case must be single molecules the probability of single molecule infections will be $px_0 e^{-px_0}$. The expected percentage of single molecule infections is recorded in fig. 5 (curve A) together with the data from table XII. The calculated rate of infections of this kind is obviously far too high to fit the experiment.

Alternative B. It will be shown later that the molecules of the virus protein are in all probability rodshaped. It is, therefore, likely that

association of molecules, if occurring, would be principally end to end as usually happens with molecules of that shape. If we denote the ends of a molecule a and b and suppose that the intermolecular affinities go from a to b, we can signify a chain of molecules with the symbol $ab—ab—ab$. In this chain all bonds are of the same kind, and in case of a dissociation they will all have the same chance to break, i.e. the dissociation constants of 1, 2, 3, etc. order would be equal. Let x_n signify the concentration of chains containing n molecules. If k be the dissociation constant we have

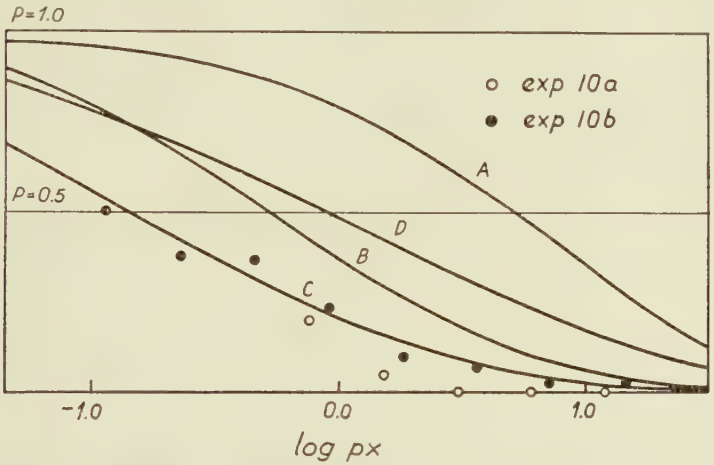

Figure 5. Probability of single molecule infections. Alternatives *A*—*D*. Experiment 10.

$$x_2 = x_1^2/k$$
$$x_3 = x_1 x_2/k = x_1^3/k^2$$
$$\cdots\cdots\cdots$$
$$x_n = x_1^n/k^{(n-1)}.$$

Let x be the total concentration of virus and x_0 the concentration of aggregates and we have

$$x = x_1 + 2x_1^2/k + 3x_1^3/k^2 + \cdots + n x_1^n/k^{(n-1)} + \cdots$$
$$x_0 = x_1 + x_1^2/k + x_1^3/k^2 + \cdots + x_1^n/k^{(n-1)} + \cdots$$

This leads to

$$x = x_1 : (1 - x_1/k)^2 \qquad (19\,a)$$
$$x_0 = x_1 : (1 - x_1/k). \qquad (19\,b)$$

And finally

$$x_0 = \frac{k}{2}\left(\sqrt{1 + \frac{4x}{k}} - 1\right) \qquad (19)$$

$$x_1 : x_0 = x_0 : x. \qquad (19\,c)$$

The formula for calculation of the number of active particles will, thus, be exactly the same as the function (*18*), and the distribution curve consequently identical with curve A fig. 4. The number of single molecule infections will, however, be different. According to function (*19 c*) the ratio of such infections will be $\frac{x_0}{x} p x_0 e^{-p x_0}$. The corresponding curve is found in fig. 5 (curve B). Although this alternative gives a lower rate of infections of this kind it is still too high to fit the experiment.

Alternative C. If on the other hand the intermolecular bonds go from *a* to *a* and from *b* to *b*, so that a chain of molecules would be signified by the symbol *ab—ba—ab—ba*, there will be two different kinds of bonds with different dissociation constants. Chains of a certain odd number of molecules will all be of the same kind with one free *a* pole and one free *b* pole. Chains of an even number of molecules will belong to either one of two types, with two free *a* or two free *b* poles. Let the concentration of the different kinds of aggregates be x_1, $(u_2 + v_2)$, x_3, $(u_4 + v_4)$, etc. Let further the number per unit volume of free poles be F_a and F_b, and the number of bonds B_a and B_b, and we have

$$F_a = x_1 + 2u_2 + x_3 + 2u_4 \cdots \quad F_b = x_1 + 2v_2 + x_3 + 2u_4 + \cdots$$

$$B_a = v_2 + x_3 + 2v_4 + 2x_5 + \cdots \quad B_b = u_2 + x_3 + 2u_4 + 2x_5 + \cdots$$

The rate of dissociation will be proportionate to B_a and B_b. The rate of association will be proportionate to the number of possible combinations of free poles, i.e. to $F_a^2/2$ and $F_b^2/2$.[1] Letting the dissociation constants be k_a and k_b, we get the following system of equations, x_0 and x having the same signification as before:

[1] The number of possible combinations is $\frac{F(F-1)}{2}$. The above approximations can be made as F_a and F_b have large numerical values.

$$\begin{cases} 2\,k_a\,B_a = F_a^2 \\ 2\,k_b\,B_b = F_b^2 \\ F_a + F_b = 2\,x_0 \\ 2\,B_a + F_a = 2\,B_b + F_b = x. \end{cases}$$

This leads to

$$x_0 = \frac{k_a}{4}\cdot\left(\sqrt{1+\frac{4x}{k_a}}-1\right)+\frac{k_b}{4}\cdot\left(\sqrt{1+\frac{4x}{k_b}}-1\right). \qquad (20)$$

The deduction of the number of single molecule infections can be made in the following way. Using the same symbols and remembering that the same chain with an odd number of molecules is formed regardless whether a single molecule is added to a u- or a v-molecule, we have the following series of equilibriums:

$$u_2 = \frac{x_1^2}{2\,k_a}, \qquad v_2 = \frac{x_1^2}{2\,k_b},$$

$$x_3 = \frac{x_1\,v_2}{k_a}+\frac{x_1\,u_2}{k_b} = \frac{x_1^3}{k_a\,k_b},$$

$$u_4 = \frac{x_1\,x_3}{2\,k_a} = \frac{x_1^4}{2\,k_a^2\,k_b}, \qquad v_4 = \frac{x_1\,x_3}{2\,k_b} = \frac{x_1^4}{2\,k_a\,k_b^2},$$

$$x_5 = \frac{x_1\,v_4}{k_a}+\frac{x_1\,u_4}{k_b} = \frac{x_1^5}{k_a^2\,k_b^2}$$

etc.

We get, therefore:

$$x = x_1 + x_1\cdot\left(\frac{x_1}{k_a}+\frac{x_1}{k_b}\right)+3\,x_1\cdot\frac{x_1}{k_a}\cdot\frac{x_1}{k_b}+2\,x_1\cdot\frac{x_1}{k_a}\cdot\frac{x_1}{k_b}\cdot\left(\frac{x_1}{k_a}+\frac{x_1}{k_b}\right)+\cdots$$

$$x_0 = x_1 + 1/2\,x_1\cdot\left(\frac{x_1}{k_a}+\frac{x_1}{k_b}\right)+x_1\cdot\frac{x_1}{k_a}\cdot\frac{x_1}{k_b}+$$

$$+\,1/2\,x_1\cdot\frac{x_1}{k_a}\cdot\frac{x_1}{k_b}\cdot\left(\frac{x_1}{k_a}+\frac{x_1}{k_b}\right)+\cdots$$

This leads to

$$x = x_1 \cdot \frac{1 + \frac{x_1}{k_a} + \frac{x_1}{k_b} + \frac{x_1^2}{k_a k_b}}{\left(1 + \frac{x_1^2}{k_a k_b}\right)^2} \qquad (20\ a)$$

$$x_0 = 1/2\, x_1 \cdot \frac{2 + \frac{x_1}{k_a} + \frac{x_1}{k_b}}{1 - \frac{x_1^2}{k_a k_b}}. \qquad (20\ b)$$

If $k_a = k_b$ the functions (*20*), (*20 a*), and (*20 b*) will be identical with the corresponding expressions (*19*), (*19 a*), and (*19 b*), as must be the case.

There is an indefinite number of pairs of k_a and k_b that will equally well fit the distribution of frequencies of takes observed. There is, however, only one pair of values that simultaneously gives the best agreement with both the total number of takes and the number of single molecule infections. The curves C in fig. 4 and 5 correspond to $pk_a = 0.03$ and $pk_b = 1.5$. There is a fair agreement with the experimental data. χ^2 amounts to 17.53 with a probability of identity of 0.1—0.2, thus slightly more than in the cases A and B, but still within the limits of a chance variation. Curve C is the only one to fit acceptably with the number of single molecule infections.

Alternative D. There is, however, another line to follow in the search for an explanation of the experimental data. If there is a definite variation in the value of p, i.e. if the groups of test animals are heterogeneous with regard to resistance, there will be a deviation from the normal distribution of takes. Let $p_1, p_2 \cdots p_n$ denote the different p values in a group of n animals. One can easily see that the corresponding probability of takes will be

$$P = 1 - \frac{S\,(e^{-p_n x})}{n}. \qquad (21)$$

The corresponding distribution curve will be the average of a series of normal curves, provided that all test groups are similar with regard to the average p value and the variation around it. It is immediately evident that a curve of that type must have a flatter course than the normal curve. As an example a curve of this kind is shown in fig. 3, (curve D). For the construction of this curve, log p was chosen as a

measure of the resistance, and it was further assumed that the frequency distribution was of the normal type, the standard deviation being $\sigma_{\log p} = 0.6$. This means that in a group of 48 animals the following classification of resistance could be made:

1)	a group of	3	animals	with average	$\log (p_n/p)$	$= -1.2$
2)	»	12	»	»	»	$= -0.6$
3)	»	18	»	»	»	$= \pm 0$
4)	»	12	»	»	»	$= + 0.6$
5)	»	3	»	»	»	$= + 1.2$

The resulting curve fits excellently with the experimental data, χ^2 being but 13.31 with a probability of identity of 0.3—0.5. The probability of single molecule infections will be

$$P_1 = \frac{S\,(p_n x \cdot e^{-p_n x})}{n}. \qquad (22)$$

The corresponding curve is shown in fig. 5 (curve D). In this respect the fit is not good.

It is impossible to draw any definite conclusions on the basis of the above discussion. In important points the reasoning was based upon the theory of single molecule infections. Although there is much in favour of that assumption, it is nothing but a hypothesis, and until further evidence is collected, proper caution must be exercised. In the following discussion it must be remembered that B and C are the only alternatives to exclude each other. Each one can exist in combination with A, or D, or both. Whichever the combination, the alternative D must be expected to play a part, simply for the reason that absence of individual variation would be contrary to all experience. Further evidence to that end can be obtained from table IX. A distribution curve, based upon one of the functions (*18*), (*19*), or (*20*) has a slope that to a certain extent is dependent upon the value of pk. As k must be assumed constant for a given virus and a given solvent, the slope will depend upon p. With decreasing p the functions (*18*), (*19*), and (*20*) give distribution curves ever more approaching the limit function (*23*), where K has the form $p\sqrt{k}$. The slope of the curve

$$P = 1 - e^{-K \cdot \sqrt{x}}. \qquad (23)$$

represents, therefore, the lower limit of the corresponding distribution curves. Function (*23*) is shown in fig. 4. The slope of the approximately straight portion is 0.39 per unit of log *px*. Now we have in exp. 8 table IX, the age group *a* with a distribution curve, the slope of which is but 0.125. This type of flat curve cannot be explained on the basis of functions (*18*), (*19*) or (*20*) exclusively. Function (*21*) on the other hand can assume any degree of slope from 0 to that of the normal distribution curve. As a matter of fact we must expect a considerable heterogeneity in age group *a*, as it comprises animals from 54 to 92 days old.

A combination of variation in host resistance and aggregation of virus particles seems to be most fit to explain the results in table XII. It is not even necessary to assume a reversible aggregation. For it must be remembered that the frequency figures in the last column of table XII are at least uncertain, considered the small number of observations. A reduction in the values of curve D, fig. 5, with a constant fraction might therefore very well fit the experiment.

It is hard to say whether the observations by Bald on the tobacco mosaic virus could be interpreted in the same way. In that case one must obviously assume the existence of entry points with different degrees of susceptibility on the surface of one and the same leaf. Although this seems *a priori* not unprobable, to the writer's knowledge there are so far no direct observations on this matter.

Table XII can also serve as basis for a further analysis of the incubation time method. Let t_x denote the incubation period after infection with x virus particles, and we can assume the following conversion of the formula (*7*)

$$\frac{1}{t_x} - \frac{1}{t_1} = b \cdot (\log x - \log 1)$$

b having the same numerical value as in (*7*), but being positive. From table XII the value of $\frac{1}{t_1}$ was estimated to 0.055 ± 0.004. Previously the numerical value of b was calculated to 0.0547 ± 0.0008. It seems, therefore, very reasonable to assume $b = \frac{1}{t_1}$ and simplify the formula to

$$\frac{1}{t_x} = b \cdot (\log x + 1). \qquad (24)$$

In a sufficiently large group of animals inoculated with equal amounts of the same virus dilution the fractions infected by 1, 2, 3 etc. virus particles will be

$$P_1 = p\,x \cdot e^{-p\,x}$$

$$P_2 = \frac{(p\,x)^2}{2!} \cdot e^{-p\,x}$$

$$P_n = \frac{(p\,x)^n}{n!} \cdot e^{-p\,x}.$$

If $\bar{y}$ is the average of $\frac{1}{t}$ through the whole group of animals we have according to formula (*24*)

$$\bar{y} = b \cdot [P_1 (\log 1 + 1) + P_2 (\log 2 + 1) + \cdots\cdots]$$

provided, that b is constant, i.e. all animals of uniform resistance, and that each particle contains only one virus molecule. But

$$P_1 + P_2 + P_3 + \cdots = 1 - e^{-p\,x}.$$

Thus

$$\bar{y} = b \cdot (1 - e^{-p\,x}) + b \cdot S\left[\frac{(p\,x)^n \cdot \log n}{n!} \cdot e^{-p\,x}\right]. \qquad (25)$$

Function (*25*) has two asymptotes. When px decreases towards 0 the function will approach »from above» the value $b\,.\,(1—e^{-px})$. For large px the first term will assume the value b and the second term approach »from below» the value $b.\ \log\ px$. From values of px of 10 and upwards function (*25*) will, therefore, be practically identical with function (*24*). In fig. 6 the theoretical curve is compared with the experimental data from table XII. The agreement is satisfactory.

If the particles in the virus suspension do not consist of single virus molecules but are aggregates of a constant number of c molecules, formula (*25*) will assume the form

$$\bar{y} = b \cdot (1 + \log c) \cdot (1 - e^{-p\,x/c}) + b \cdot S\left[\frac{(p\,x)^n \cdot \log n}{n!} \cdot e^{-p\,x/c}\right].$$

For large px values this function will have the same value as (25) For small px on the other hand $\bar{y}$ will be significantly lower than in case (25). In case of a reversible dissociation of the aggregates with dilution, therefore, no appreciable deviation from the formula (25) can be expected.

In order to compute the influence of variation of resistance upon the incubation time curve one has to consider that b certainly varies with p. As long as the type of this covariation is not known, it is

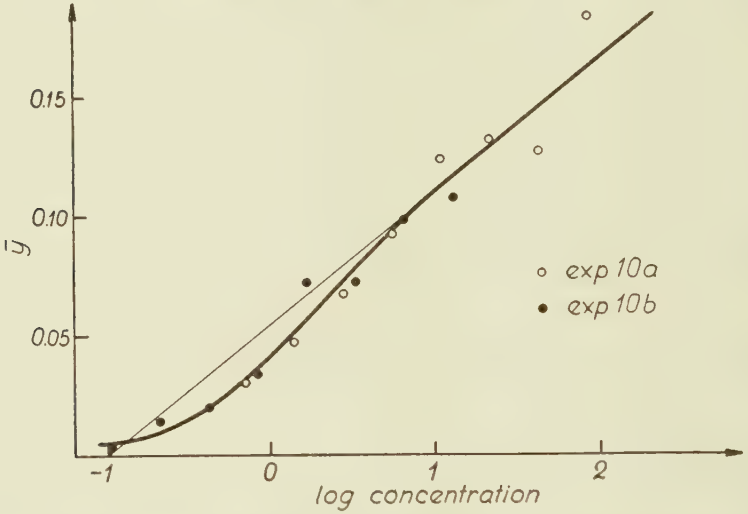

Figure 6. Experimental data fitted to the theoretical incubation time curve. Experiment 10.

futile to speculate over the details of the curve to be expected as the result of such a variation. On the whole, however, it can be constructed like the function (21) as an average of a series of essentially similar curves with different b and p. It will, therefore, largely be of the same type although the way in which it approaches the asymptotes and the slope of the rectilinear part will depend upon the covariation of b and p.

In the above discussion it was tacitly assumed that more than one virus molecule can enter the same cell, and incite a process of the same course as after as many single hits in different cells. This assumption is by no means self-evident. The phenomenon of interference between different strains of the same virus as exhibited

for instance in yellow fever, must be interpreted as the result of a competition about the susceptible cells. Once a virus particle has entered the cell the affinities are saturated and a superinfection by further virus particles is impossible. As will be shown later there seems to exist no interference in mouse poliomyelitis, indicating that superinfection is possible. From this point of view the reasoning should, therefore, be correct. On certain theoretical grounds to be discussed later one might, however, expect that the course of the infection after a certain number of hits will be different, depending upon the number of cells concerned. At present the experimental evidence towards that end is insufficient, and the analysis cannot be extended further.

The agreement between found and expected as illustrated in fig. 6 does not, of course, prove the validity of the formula (*24*), but shows that if this function is valid, it can be applied to the average from a group of animals as well. A comparison between fig. 6 and the different types of curves previously discussed reveals the following facts. The point $px = 1$ corresponds to log dilution 6.96 in the incubation time curve and 6.98 in the distribution curves A, B, and C fig. 4. As these two estimates of the virus concentration are largely independent of each other, the close agreement must be regarded as suggestive evidence of the validity of the formula (*24*). In the distribution curve D, $px = 1$ corresponds to log dilution 6.56. This figure is, however, not compatible with the incubation time titer as being based upon different assumptions, and the numerical values now mentioned cannot serve as an evidence in favour of one of the alternatives A—C.

The 50 p. c. point corresponds to log $px = + 0.24$ in curves A and B and to log $px = + 0.29$ in curve C. In conformity with these figures the average from a number of titrations listed in table XI was calculated to $+ 0.277$.

According to the above discussion the 50 p. c. endpoint titer as a measure of the activity is decidedly unsatisfactory. Its relationship to the actual virus concentration is dependent not only upon the resistance of the test animals but also upon the physical state of the virus particles. The incubation time method on the other hand seems to be largely independent of the latter condition and, therefore, to give a more reliable information about the virus content. In the

present case, therefore, it seems to be more logical to connect the definition of an infective unit with the latter method. Consequently the following definition is formulated.

DEFINITION. *One MID of the encephalitogenic strains of mouse poliomyelitis virus contains a number of* $\frac{1}{p}$ *virus molecules,* p *being the resistance factor of the probability function. The activity of a given virus solution is the number of MID per ml.*

For calculation of the activity one has to use the formula (*24*). Let A be the activity. The standard volume of the inoculum is 0.02 ml. Consequently we have

$$\bar{y}_0 = b \cdot (\log 0.02\, A + 1)$$

$$\log A = \frac{\bar{y}_0}{b} + 0.7 \qquad (26)$$

$\bar{y}_0$ denoting the reciprocal of the harmonic mean of the incubation periods obtained after inoculation of the undiluted material and b having the value 0.0547. All titer values recorded in the following were calculated according to this formula.

* * *

It was previously pointed out that the correlation between the amount of virus inoculated and the length of the incubation period led to the assumption that the rate of multiplication of virus was "overlogarithmic". An attempt to visualize the mechanism of this process was made on the basis of the following reasoning.

A logarithmic rate of multiplication will be the result if each virus particle through branching or otherwise gives rise to a constant number of daughter particles and the generation period is constant throughout the growth phase. Let the number of daughter particles be m, the generation period t, y the number of descendants of one single virus particle at the time x, and we have

$$y = m^{x/t}$$

or

$$\log y = \frac{\log m}{t} \cdot x.$$

The rate of multiplication will be given through the expression

$$\frac{dy}{dx} = \frac{\log m}{t} \cdot y. \qquad (27)$$

On page 37 it was pointed out, that the overlogarithmic type of multiplication in the case of mouse poliomyelitis could best be explained by the assumption of the existence of a gradually decreasing inhibitory influence. It would be only logical to look for the origin of that inhibitory effect in the ganglion cells themselves. It seems not unreasonable to assume that an intact nerve cell would exert a certain resistance against the multiplication of virus and possibly through neural impulses induce resistance in other cells as well. With invasion and multiplication of virus the resistance would gradually break down and the presumed induction of surrounding nerve cells would cease to exist. According to this hypothesis the rate of inhibition would be roughly proportionate to the number of intact cells.

There are some observations in favour of this theory. Howe and Bodian (68, 69) found that lumbar cord cells in the stage of chromatolysis or "axone reaction" after transsection of the sciatic nerve were resistant to infection with poliomyelitis virus. This refractivity was only relative and most pronounced in compact groups of chromatolytic cells. In such groups even normal, non-chromatolytic cells were sometimes spared. On the other hand scattered individual chromatolytic cells were more likely to succumb. Howe and Bodian interpret this phenomenon as caused by lack of suitable substrate for the virus, i.e. by a deficiency in activating factors. It might, however, as well or better be conceived as an increase in the concentration of inhibiting substances. The stage of repair no doubt involves a metabolic hyperactivity, during which the hypothetical inhibitory effect on the multiplication of virus might be increased and also transmitted to cells in the close vicinity, so that even normal cells surrounded by larger numbers of chromatolytic cells are sometimes resistant.

Furthermore some of the observations already reported are in conformity with the hypothesis. If a certain number of virus molecules are distributed on one occasion over a small amount of tissue, on another over a wider area, the actual number of hits would be the same, as the chance of hit is constant. The number of invaded

cells, however, would be larger in the latter case, i.e. the inhibiting influence less pronounced, and one would accordingly expect a more rapid multiplication. This is in full agreement with the results in table V, illustrated in fig. 1, these experiments showing that application of large inocula gave disproportionately short incubation periods. — If on the other hand the ratio susceptible cells /resistant cells decreases, as must be the case in adult mice, the rate of multiplication of virus would decrease, in accordance with the experimental data (tables VIII and IX).

In the formula (*27*) m can be said to represent the type of biological or chemical process, significant for the propagation of the virus in question, while t is a measure of the rate of multiplication, i.e. m is a characteristic of the virus, t one of the host. An inhibitory effect from the side of the host would be most likely to cause a change of t, while m would be unaffected.

Suppose that the susceptible cells are evenly distributed over a sphere, the radius of which is unit. In the centre the inoculum is deposited, spreading over a concentric sphere with the radius r. From the site of inoculation the virus migrates evenly in all directions, the rate of migration being v. The number of intact cells at the time x after inoculation will then be proportionate to $1 - (r + vx)^3$, and the rate of inhibition will be a function of this expression. r is an arbitrary constant, dependent upon the conditions of the experiment and v might be a constant or be a function of r and x. Let t be the generation time in the case, that all susceptible cells are invaded, and the rate of multiplication can be expressed as follows.

$$\frac{dy}{dx} = \frac{\log m}{t + f[1 - (r + vx)^3]} \cdot y. \qquad (28)$$

At present it is impossible to give any hints as to the actual form of this function. If, however, the rate of inhibition is a linear function of the number of intact cells and the rate of migration of the virus is constant, the formula could be given the following form

$$d(\log y) = (C_0 + C_1 x + C_2 x^2 + C_3 x^3 + \cdots) \cdot dx \qquad (28\,a)$$

C_0, C_1, C_2, etc. being constants.

According to the reasoning on page 36 the formula (*24*) leads to the following growth equation

$$\log y = \frac{bx}{1 - bx}. \qquad (29)$$

By differentiating we get

$$d(\log y) = \frac{b}{(1 - bx)^2} \cdot dx = (b + 2b^2x + 3b^3x^2 + \cdots) \cdot dx. \quad (29a)$$

In the formulas (*28a*) and (*29a*) the series of coefficients C_0, C_1, C_2, ... and b, $2b^2$, $3b^3$, ... are rapidly converging towards 0. For small values of x, therefore, all terms from a certain $C_n x^n$ are in-

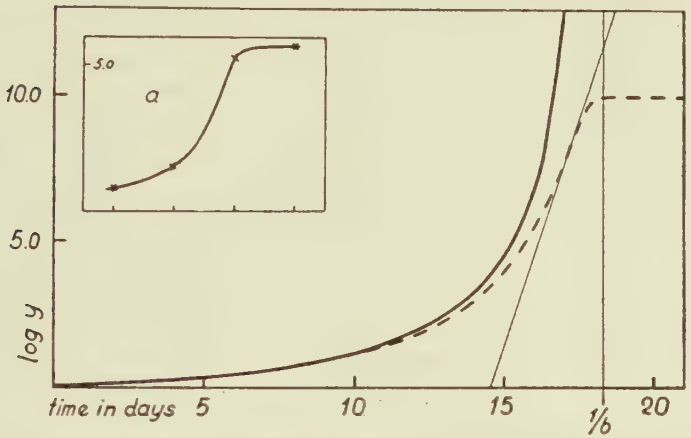

Figure 7. Multiplication of virus, theoretical curves. Inserted (*a*) experimental data from Theiler and Gard.

significant. It is, therefore, possible to identify (*28a*) with (*29a*) for small values of x by arbitrarily making a number of the coefficients C_n identical with the corresponding ones in the formula (*29a*). The coefficients C_n, however, are functions of the five unknown quantities in the formula (*28*), and therefore not more than five of them could be arbitrarily chosen. Thus it is possible to find values of the constants such as to give $C_0 = b$, $C_1 = 2b^2$, $C_2 = 3b^3$, $C_3 = 4b^4$, and $C_4 = 5b^5$, while the rest of the coefficients C_n might differ from the corresponding nb^n. Formula (*28a*) will then be identical with (*29a*) at least up to the point, where the term $6b^6x^5$ begins to be significant.

The growth curve corresponding to the formula (*29*) is shown in fig. 7 (the heavy line). For $x = \frac{1}{b}$ log y as well as $\frac{d(\log y)}{dx}$ are in-

finite. This can hardly agree with the experimental data. On the contrary it was previously pointed out that the concentration of virus never exceeds a certain limit. For $x = \frac{1}{b}$, therefore, log $\bar{y}$ should assume a certain well defined finite value. It is also obvious that the rate of multiplication of virus must have a finite value. On page 54 it was pointed out that the method used for calculation of the incubation period gives but approximately correct figures. From this fact it is evident that the formula (*29*) gives only an approximation of the real growth function. The corrections to be instituted on behalf of the approximated calculation of the incubation period lead to a displacement of the curve towards larger x values. The dotted line in fig. 7 is an attempt to illustrate the probable course of function (*28*). The flex tangent (fine line) denotes the maximum rate of multiplication. When this point is reached all susceptible cells are invaded and multiplication can proceed only till the virus concentration of each cell has reached the limit. In fig. 7 a further diagram is inserted, based upon an experiment by Theiler and Gard (Jour. Exp. Med. *72*, p. 60, table V), in which the virus content of the brain was determined at intervals after inoculation. Although no direct comparison as to numerical values is possible, it is obvious that the multiplication of virus really follows a curve of the general type now described.

This discussion was not aimed at solving the problem. The writer's intention has been to present a general line of thought along which the solution might possibly be found. As to the nature of the inhibitory factor and the transmission of the effect from one cell to another nothing but a guess can be advanced. It might be a substance produced in the cells and excreted, or it might be conceived as a neurally transmitted mutual control of the metabolism, a sort of trophic tonus.

CHAPTER V.

Purification of neurovirus.

When Stanley (142) in the year 1935 obtained a crystallizing protein, showing all the characteristics of tobacco mosaic virus, a new epoch in the history of virology had started. Not that the purification of viruses with the aid of chemical methods was a new idea. Attempts to that effect had been made for about a decade. Neither was Stanley's protein the first pure virus preparation, elementary bodies of vaccinia being prepared in more or less pure state by Mac Callum and Oppenheimer in 1922 (101). Stanley, however, purified a virus with elementary particles of such a small size as to be classified as molecules. His investigations proved that this virus in pure state acted as a molecular chemical compound, as a protein. Viruses were regarded as minute microorganisms and as such in some mysterious way standing above the laws of chemical substances. On that account Stanley's result was felt like a shock. Later, however, it was realized that it was not conclusive evidence against the vitalistic theory.

Stanley's work incited numerous attempts to purify viruses of different kinds, principally plant viruses. His results were confirmed, and using the same method of purification — repeated differential precipitation with ammonium sulphate at controlled pH — Bawden and Pirie (8, 9, 10) were able to obtain in pure state the cucumber viruses 3 and 4 and the viruses of latent mosaic and bushy stunt.

In the case of less stable viruses, however, this procedure was not satisfactory, as the material was largely inactivated during the purification process. Attempts were made, therefore, to find less drastic methods. MacCallum and Oppenheimer had found that the virus of vaccinia could be purified by means of differential centrifugation. This method was later successfully used by Ledingham (97) and further elaborated by Parker and Rivers (114). At the same time the

quantity high speed centrifuge had been largely improved by Gratia (55) and especially by Beams and Pickels (11). It was now possible to subject comparatively large quantities of material — up to 100 ml — to centrifugal fields of more than 50 000 times gravity at a low temperature, the latter not the least important when treating unstable organic material. The new equipment was tested in purification experiments and proved to yield excellent results. Thus it was possible to obtain in pure state the viruses of tobacco ring spot and severe etch and the cucumber virus 1. (144).

Animal viruses, usually being less stable than plant viruses, have proved more difficult to purify. The chemical methods have generally not yielded satisfactory results. By means of differential centrifugation, however, supposedly pure preparations of the viruses of vaccinia, Rous' sarcoma (25), Shope's rabbit papilloma (12), and equine encephalitis (162) have been obtained.

Janssen (74) elaborated for the virus of foot and mouth disease a purification procedure that differs principally from the usual methods. Adsorption and elution as used in the enzyme chemistry had frequently been tested in experiments on viruses. It was, however, found a matter of difficulty to elute the virus without destruction of the activity. Janssen, therefore, aimed at a method by which the adsorbent could be manufactured in the reaction mixture, centrifuged off and redissolved, thereby making the virus free. He found that a virus containing fluid, saturated at room temperature with calcium sulphate, on addition of 20 p. c. alcohol or a mixture of alcohol and ether, formed a precipitate containing all the activity and but little extraneous material. This precipitate could be redissolved in water and the process repeated deliberately. The final preparation consisted chemically of a nucleoprotein and was considered by Janssen to be pure virus. He also attempted later to purify the virus by means of differential centrifugation (75).

Attempts at purification of the virus of poliomyelitis have been made now and again. Howitt (72) found that infectious spinal cord could be defatted with ether and that a considerable amount of extraneous proteins could be removed from the remainder by precipitation with basic lead acetate, without much loss of activity. Clark (23, 24) and his collaborators concentrated virus containing extracts by means of vacuum distillation, removed the excess of salts in dialysis and precipitated the virus with ammonium sulphate.

They found, that the virus was completely precipitated in 50 p. c. saturated solution but remained largely in the supernatant at 30 p. c. saturation. If the material had been extracted with ether before the precipitation, the virus was practically insoluble in distilled water. They found finally, that the virus could be concentrated by means of centrifugation at high speed.

Rhoads (126) had shown that the virus could be adsorbed on aluminum hydroxide. Sabin (128) found that the activity could be eluted in quantity from the precipitate with dilute disodium phosphate solution. After subsequent dialysis and vacuum distillation he obtained a preparation giving negative tests for protein and ten times as active as the starting material. Behrens and Barker (13) accomplished a partial purification by means of repeated isoelectric precipitation at pH 4.8—5.0 with 0.01 m citric acid.

Brown and Kolmer (16) used differential precipitation with ammonium sulphate according to Stanley and obtained a solution practically retaining the original activity. It flocculated sharply at pH 4.0. The precipitate could be dissolved in water and reprecipitated at the same pH. The final solution contained but 0.01 p. c. protein.

I. Neurovirus from mice.

The principal difficulty involved in the purification of brain and cord suspensions, is the presence of large amounts of lipoid matter. These substances are easily emulsified even in dilute protein solutions. On account of blocking they make filtration of the extracts practically impossible. They have a low specific gravity and cannot, therefore, be removed by centrifugation. This difficulty was encountered by Beard and his collaborators (37) purifying the virus of equine encephalitis from chick embryos. They observed serious disturbances of the purification process unless the brain and cord were removed from the embryos before preparation of the extracts.

If a brain suspension is shaken with half the volume of ether, the mixture forms a homogeneous emulsion. On standing, however, it soon separates into two or three distinct layers: a top layer, consisting of ether and ether soluble substances, a middle layer containing lipoids and lipoproteins, insoluble in ether and water, and an aqueous bottom layer with the water soluble substances. This aqueous solution, however, still contains large amounts of particulate matter of low

specific gravity, making further purification by means of differential centrifugation impossible.

In an attempt at concentration and partial purification of poliomyelitis virus from sewage and stools the present writer employed precipitation with ammonium sulphate (46). With the aim to obtain the virus fraction in an easily separable form the precipitation was made in the presence of ether. The top layer was found to contain the whole precipitate. When the top disc was dialysed or extracted with water, it was observed that the extract remained water clear as long as a sufficient amount of ether was present but turned opalescent or turbid as soon as the ether was removed.

This observation was used by Gard and Pedersen (50) in purification experiments on mouse poliomyelitis virus from infected mouse brain. A brief account of these experiments has previously been published. In the following they will be described in detail together with the subsequent work.

Preparation of extracts.

First it was attempted to determine the most favourable conditions for extraction of the tissue.

Experiment 11. 18 brains were ground with sterile sand and 15 ml of distilled water. 2 ml portions of the heavy suspension were thoroughly mixed with 8 ml of buffer solution (ionic strength = 0.1). After 2 hours at + 4° C the mixtures were centrifuged at low speed, the supernatants removed and diluted 1 : 100 and 1:10 000. Of each dilution 0.02 ml amounts were inoculated intracerebrally to groups of 5 mice. The results are summarized in table XIII.

Table XIII.

Influence of pH at the extraction of virus containing nerve tissue.

Buffer	pH of extract	titer of extract = log A
Acetate	5.1	7.8
Phosphate	6.1	7.4
»	7.1	7.7
Borate	7.8	7.9
»	9.0	8.3
»	9.7	8.1
Dist. water	7.3	8.1

The differences observed are not statistically significant. There is, however, a certain trend towards an optimal pH at about 9. This coincides fairly with the range of maximum stability of the virus. The differences might, therefore, well be regarded as a pH effect on the stability rather than the solubility of the virus.

In similar experiments the effect of different salt concentration of the diluent — from 0 to 2 m sodium chloride solution — was studied. No significant differences were observed. It was found, however, that the coarse material of the suspensions showed a maximum sedimentation velocity in the range between 0.025 and 0.075 m NaCl.

The quality of the extracts was to a certain degree dependent upon the quantity of diluent used. If 10 p. c. suspensions were left to settle over night in the cold room, the bottom layer of coarse material usually amounted to more than 50 p. c. of the total volume, and the supernatant was very turbid. In 5 p. c. suspensions the bottom layer was less than 20 p. c. and the supernatant much clearer. Further dilution had no significant effect.

Experiment 12. The completeness of the extraction was studied in the following experiment. 500 brains were ground with sand and made up with diluent to a total volume of 3 500 ml. After settling over night in the cold, 2 750 ml of supernatant was siphoned off, and further diluent added to the remainder up to the original volume of 3 500 ml. A second extract of 3 000 ml was collected. The activity of the extracts was tested and found to be log A = 6.35 and 5.36 respectively. Thus, the first extract contained 90 p. c. of the total activity.

In accordance with the above experiments the crude extracts were prepared as follows. The brains were thoroughly ground with sand and mixed with a diluent consisting of 0.05 m sodium chloride solution to which was added phosphate buffer of pH 8.0 to an ionic strength of $\mu = 0.001$. The total volume was adjusted to give a 5 p. c. suspension, calculated on brain wet weight. The mixture was allowed to settle in high glass cylinders over night in the cold room. The supernatant extract was then siphoned off and the remainder discarded.

Removal of the lipoidal constituents.

In the crude extracts rather large quantities of material were sedimentable at high speed centrifugation. Part of this sedimented also at low speed, and during repeated fractionation in the centrifuge

an increasing amount of coarse material could be removed. When analysed in the ultracentrifuge the purified remainder, however, proved to be inhomogeneous, without any distinct sedimentation boundaries, regardless of the number of fractionations. There seemed to be no end to this purification procedure. If differential centrifugation was to be employed, which from several points of view was favourable, the crude extracts must obviously undergo some initial treatment in order to remove the mass of lipoidal matter.

Since desiccation in vacuo destroys the activity, extraction of the dried material with benzene and other lipoid solvents was out of the question. As already mentioned separation with ether according to the three-layer-method of the aqueous extracts led to a considerable purification, although enough lipoids were left in solution to make further purification by means of differential centrifugation a tiresome business. It was, therefore, attempted to get more suitable extracts by means of enzymatic digestion of the macerated brains.

Experiment 13. In four experiments infected brains were ground with sand and small amounts of water. Parts of the heavy suspensions were mixed with 3 or 4 volumes of equal parts of pancreatic juice and glycerin. A set of controls were mixed with 50 p. c. glycerin and their pH adjusted to that of the reaction mixtures. The samples were kept at 37° C for 16—36 hours. They were then centrifuged at low speed and the supernatants titrated in mice. The results of these tests are summarized in table XIV.

Table XIV.

Influence of digestion with pancreatic juice on the activity of infected mouse brains.

Time		Titer = log A	Difference
36 hrs.	Digest	8.09	+ 0.79
	Control	7.30	
16 hrs.	Digest	7.49	+ 0.65
	Control	6.84	
24 hrs.	Digest	7.15	+ 0.39
	Control	6.76	
24 hrs.	Digest	8.27	+ 0.62
	Control	7.65	

In all cases the digest had a higher titer than the corresponding control. The mean difference 0.61 ± 0.146 is statistically significant.

The digestion obviously did not inactivate the virus. Whether on the other hand the apparent activation should be accounted for by a preservative effect, an actual activator in the pancreatic juice, or the destruction of an inhibitor in the brain tissue remained unsettled. There are, however, facts in favour of the two latter alternatives. The digested material was subjected to differential centrifugation but proved to be even less suitable than untreated extracts.

Experiment 14. Several other procedures were tested. In preliminary experiments it was found, that a maximum of flocculation occured at about pH 4.5. The following experiment was set up. 9 brains were ground with sand and 15 ml of distilled water. The suspension was centrifuged to remove sand and coarse particles. The supernatant was divided into 4 portions, to each of which was added 1 volume of buffer solution of ionic strength $\mu = 0.1$. The mixtures were centrifuged at low speed, the supernatants poured off and sampled for pH and nitrogen determinations. 1 ml of each supernatant was neutralized against phenol red, made up to 10 ml, and titrated in mice. The results are summarized in table XV.

Table XV.

Isoelectric precipitation.

Buffer	Supernatant	pH	mg N per ml	titer
Acetate	almost clear	4.07	0.422	3.41
»	clear	4.39	0.417	2.88
»	»	4.57	0.407	2.83
Phosphate	turbid	7.19	0.789	6.61

From this it is evident, that the total activity is precipitated together with approximately 50 p. c. of the nitrogen. Clarification by this means was therefore not practicable. — In a second experiment the properties of the precipitate were studied.

Experiment 15. To a measured amount of extract acetate buffer was added to give a final pH of 4.5. The mixture was centrifuged at low speed, the supernatant poured off and neutralized. The precipitate was suspended in phosphate buffer of pH 8.0, centrifuged and made up to the original volume. Both fractions were titrated in mice together with an untreated control. The titers were: precipitate 8.00, supernatant 4.34, and control 8.00. Thus the activity was completely recovered from the precipitate. The suspension was milky and presented the same difficulties at centrifugation as did the original extract.

Janssen's purification method was tested. The virus was, however, rapidly inactivated in the concentrations of alcohol used. At a concentration of 5 p. c. alcohol the loss of activity amounted to only 10 p. c. but the precipitate formed contained less than 1 p. c. of the total activity. Other adsorbents were tested as well. Floridin was selected on that account, that it can be easily removed by sedimentation without centrifugation. None of the grades tested, however, adsorbed significant quantities of either virus or impurities.

Finally precipitation with ammonium sulphate was studied.

Experiment 16. To portions of 10 p. c. brain suspensions saturated ammonium sulphate solution was added to give final concentrations of 40, 30, and 20 p. c. saturation. 1/10 by volume of ether was added and the mixtures thoroughly shaken. They were then centrifuged at 3 000 r. p. m. for 15 minutes. In the two higher concentrations a top disc had formed and the mother liquor was clear, respectively almost clear. In 20 p. c. saturation there was no top layer, a precipitate had settled, and the supernatant was turbid. The mother liquors as well as the two top discs were made up to 100 times the original volume of extract with saline solution. The precipitate of the 20 p. c. solution was discarded. The diluted samples were titrated in mice with the results tabulated in table XVI.

Table XVI.

Precipitation with ammonium sulphate.

Salt concentration	Titer of	
	mother liquor	precipitate
40 p. c. saturation	5.08	8.39
30 p. c. »	7.85	7.97
20 p. c. »	8.65	—

At 40 p. c. saturation 99.95 p. c. of the virus was precipitated, at 30 p. c. saturation about 4/7, and at 20 p. c. only an insignificant amount.

The method for initial purification of the crude brain extracts finally adopted was as follows. The suspension was mixed with 1/2—2/3 by volume of ether and thoroughly shaken. The mixture was kept in a separatory flask in the cold room for 4—5 hours. The bottom layer was collected, 2/3 by volume of saturated ammonium sulphate solution was added, and the mixture left to settle in the cold during 4—5 hours. Then the water clear bottom layer was poured

off and discarded. The top disc, light greyish red in colour, was suspended in 3 volumes of distilled water, saturated with ether, and left in the cold room over night. The bottom layer, faintly yellow and opalescent, was collected, and the top layer resuspended in ether containing distilled water. The second extract was colourless and clear. The extraction was repeated twice more. The pooled extracts were run twice through a De Laval industrial separator used as clarificator with a centrifugal field of about 5000 times gravity. The solution was now free of ether and practically water clear. It was then concentrated by means of ultrafiltration as described by Seibert (137). In repeated tests it had been controlled, that no loss of activity occurred during filtration.

Usually lots of 500 mouse brains were prepared, yielding about 3000 ml of crude extract. The extract of the ammonium sulphate precipitate as a rule amounted to about 2000 ml, and was concentrated to 100 ml by ultrafiltration. The final product could easily be subjected to further purification by means of differential centrifugation without the disturbances previously mentioned.

Differential centrifugation.

In these experiments a Beam's centrifuge was used. The angle head could hold 16 tubes of 6.5 ml effective volume each. The centrifugal field was about 40 000 times gravity at a speed of 22 000 r. p. m. Before each run the rotor was cooled to +4° C. At the end of a run of three hours the temperature would usually not exceed +6° C. During the summer, when the moisture content of the air was high, large quantities of water condensed on the rotor during the transport from the cold room to the centrifuge, which hampered seriously the evacuation of the vacuum chamber. To avoid this the rotor was enveloped in a slip-cover of watertight material with a zipper, that could rapidly be removed immediately before the vacuum chamber was closed. This simple device served the purpose satisfactorily.

Experiment 17.

a) A 10 p. c. crude suspension of infected brains was spun in two experiments for 1 and 2 hours at 22 500 r. p. m. In both cases a yellowish brown, almost clear pellet had settled. The supernatants were pipetted off and the pellets resuspended in the original amount of diluent. Both fractions, supernatants and precipitates, were etherized, stored in the icebox till the following day and titrated in mice. The titers were:

1 hour	supernatant 4.37	precipitate	5.97
2 hours	4.39		6.08

Thus in both cases about 98 p. c. of the activity was found in the precipitate.

b) In another experiment a crude extract was spun 1 hour at 22 500 r. p. m. The supernatant was poured off and the precipitate resuspended in 1/10 of the original volume. After etherization the concentrate was titrated together with an untreated control. Titer of concentrate: 7.96, control: 6.80. Thus an apparent gain of activity of about 45 p. c. had occurred. The difference was, however, not statistically significant.

c) A 10 p. c. suspension was spun at 11 000 r. p. m. for 1/2 hour. The precipitated material forming a yellowish brown pellet was discarded, the supernatant after etherization titrated together with an untreated control. Titers: supernatant 7.73, control 7.50. In this case also an apparent gain of activity, about 70 p. c., had occurred.

These preliminary experiments showed that the virus under the prevailing conditions sedimented completely in 1 hour in a field of 40 000 times gravity, whereas only insignificant quantities settled during 1/2 hour in a field of 10 000 g. Accordingly the following purification procedure was tested.

Experiment 18. A 10 p. c. suspension was spun in an angle centrifuge at 3000 r. p. m. for 1/2 hour. The turbid supernatant was centrifuged at 22 500 r. p. m. for 2 hours. The supernatant was poured off = S1. The pellets were resuspended in diluent and spun at 11 000 r. p. m. for 1/2 hour. The sediment was taken up into diluent to the original volume = B2. The supernatant = S2, after sampling for activity test, was spun once more at 22 500 r. p. m. for 2 hours. The supernatant from this run = S3. The sediment was resuspended and spun at 11 000 r. p. m. for 1/2 hour. The resulting fractions = S4 and B4. All preparations were yellowish in colour and somewhat opalescent. They were titrated in mice together with an untreated control. The result is summarized in table XVII.

From this it is evident, that a great deal of the activity is lost with the fractions B2 and B4, and possibly in increasing amounts as the procedure is repeated. Another matter of interest is the fact that the total of the fractions S1, S2, and B2 shows a gain in activity of about 40 p. c. as compared with the control, whereas S3, S4, and B4 together yield the same amount of virus as did S2, from which they were derived.

The same observation was made in two of the preceding experiments, and seems to be a constant feature of the initial steps of purification. The reason is probably the presence in the crude extracts

Table XVII.

Differential centrifugation of crude brain suspension.

Fraction	Titer	Difference	Rel. conc.	Total
Control	7.34		100	100
S1	6.25	— 1.09	8	
S2	7.38	+ 0.04	110	
B2	6.70	— 0.64	23	141
S3	6.03	— 1.31	5	
S4	7.15	— 0.19	65	
B4	6.86	— 0.48	30	100

of one or two inhibitors — one that is not sedimentable and therefore separates from the virus by centrifugation at high speed, and another that is connected with the coarse material. Neither seems to combine with the virus in vitro. They are destroyed by digestion with pancreatic juice. The first one originates probably from the blood retained in the brain and may be identical with the neutralizing substance, shown by Olitsky to be present in serum of normal mice. The other inhibitor might have some connection with the tissue immunity; at least it is not present in brains of normal mice. The whole question has a bearing upon the problem of interference between different strains of virus as studied by Jungeblut and Sanders (78). Experiments along this line are carried out and will be reported in another connection. On account of the circumstances now mentioned it was considered inadequate to express the yield in terms of the activity of the crude brain suspension. In the following, therefore, all such figures refer to the virus content of extracts that have been subjected to initial purification.

Experiment 19. In order to avoid the heavy losses of activity the technique was somewhat modified in the following experiment. 350 brains were extracted as previously described, yielding 960 ml of crude suspension. This was spun at 11 000 r. p. m. for 1/2 hour. The supernatant = S1 was sampled for activity test and centrifuged at 22 500 r. p. m. for 1 hour. The precipitate was taken up into a total of 84 ml of diluent, allowed to swell in the icebox for 2 hours and then run up-and-down to 11 000 r. p. m. A small white, brittle precipitate had settled. The supernatant was fractionated in the same way twice more and successively brought down to a volume of 10 ml. All supernatants were

clear at the top with increasing cloudiness towards the bottom of the tube. All runs at the lower speed gave precipitates of the same general appearance as the first one. The final solution = S7 was titrated together with S1. The titers were S1 = 7.56, S7 = 9.30, meaning an increase in titer of 55 times with a decrease in volume of 82 times. The yield was 67 p. c. The preparation was analyzed in the ultracentrifuge and proved to be largely inhomogeneous (fig. 9).

As purification by means of differential centrifugation alone seemed to be not feasible, ether-treated brain suspension was tested as starting material.

Experiment 20. 350 brains were extracted twice. The pooled extracts were treated with ether as previously described. After dialysis against tap water the extract was centrifuged first in an angle centrifuge at 3000 r. p. m. for 15 minutes and subsequently at 11 000 r. p. m. for 1/2 hour. The supernatant = S1 had a total volume of 760 ml. It was concentrated by ultrafiltration to 220 ml. The originally waterclear extract deposited during ultrafiltration a floccular precipitate, blocking the filter. The concentrate was fractionated 5 times in the centrifuge at alternatingly 22 500 r. p. m. for 1 hour and 11 000 r. p. m. for 1/2 hour. The final solution = S11 had a volume of 6 ml, was slightly opalescent and almost colourless. A precipitate had been deposited in all low speed centrifugations although in decreasing amounts as the purification proceeded. Two of these precipitates (B3—5) were sampled for activity test, and likewise two of the supernatants from high speed runs (S8—10). The titration results are summarized in table XVIII. As demonstrated the losses of activity are definitely lower and the yield consequently higher than in the preceding experiments. Nitrogen determinations were performed on S1 = 0.351 mg per ml, and S11 = 0.069 mg per ml. Thus the activity was 8.3×10^7 MID per mg N and 4.7×10^{10} MID per mg N and the purification consequently 560 fold. — In a second experiment the yield of virus was but 25 p. c. The solution had a titer of 8.81 and the nitrogen content was 0.046 mg per ml. The activity was, therefore 1.4×10^{10} MID per mg N.

Table XVIII.

Differential centrifugation of ether treated extract.

Fraction	Titer	Rel. conc.	Volume	Yield
S1	7.48	1.0	760	
S11	9.51	107	6	85 p. c.
B3—5	8.02	3.5	6	2.8 »
S8—10	7.71	1.7	25	5.6 »

When analysed in the ultracentrifuge these preparations showed a higher degree of homogeneity, and it was actually possible to

distinguish two main components (fig. 10 and 11). The sedimentation diagrams proved, however, the state of purity to be far from satisfactory. The supposed virus component was estimated to between 6 and 16 p. c. of the total contents of solids. As furthermore, judging from the diagrams, a complete purification by means of differential centrifugation was not feasible, it was decided to add ammonium sulphate precipitation to the purification procedure.

Experiment 21. 450 brains were extracted, treated with ether, and precipitated with ammonium sulphate as previously described. The extract of the precipitate was concentrated by ultrafiltration and centrifuged at 11 000 r. p. m. for 1/2 hour. The supernatant was centrifuged at 22 500 r. p. m. for 2 hours. The precipitate from this run was resuspended in 25 ml of distilled water and kept in the cold for 12 hours. It was then centrifuged once more at 11 000 r. p. m. for 1/2 hour and at 22 500 r. p. m. for 2 hours. The final sediment, a minute, faintly yellow, clear pellet was resuspended in 1 ml of distilled water and analysed in the ultracentrifuge. Afterwards it was titrated in mice. The titer was 10.34, corresponding to a yield of 34 p. c.

The sedimentation diagram (fig. 12) showed the presence of three distinct components, which could probably be separated by means of centrifugation. As, however, one of these might be a high molecular globulin, an attempt was made to eliminate it by employing a sharpened fractionation in the initial ammonium sulphate precipitation.

Experiment 22. To 2500 ml of ether-treated extract ammonium sulphate was added to 1/6 saturation. The mixture was centrifuged at 3000 r. p. m. The top disc was removed = fraction a. The remainder was mixed with further ammonium sulphate solution to make a total concentration of 1/3 saturation and again centrifuged. The second top disc = fraction b, the clear mother liquor = fraction c. a and b were suspended in distilled water and centrifuged at 3000 r. p. m. after removal of the ether. All fractions were titrated in mice. The result is summarized in table XIX.

Table XIX.

Fractionated precipitation with ammonium sulphate.

Fraction	Volume	Titer	Yield
a	210	7.99	21.8 p. c.
b	275	8.42	77.4 »
c	3500	5.36	0.8 »

The experiment showed that 1/3 saturation was sufficient for complete precipitation of the virus. Fractionated precipitation on the other hand was not recommendable on account of the great loss of activity.

Experiment 23. In the next experiment 1100 brains were used. The ether-treated extract was precipitated at 1/3 saturation of ammonium sulphate, the precipitate washed twice with 1/3 saturated ammonium sulphate solution and finally extracted as usual. The concentrate was fractionated 4 times in the centrifuge, at 11 000 r. p. m. for 1/2 hour and 22 500 for 1 hour. The final sediment was resuspended in 1 ml of phosphate buffer solution, pH = 8.0 and ionic strength $\mu = 0.05$.

When analysed in the ultracentrifuge this preparation showed but one distinct component with no traces of impurities (fig. 13). The sediment obtained at high speed centrifugation was an almost colourless, clear, gelatinous pellet, easily soluble in distilled water and dilute salt solutions. The solution was clear with a blueish Tyndall cone. The whole preparation was used for determination of the diffusion constant, why activity test and nitrogen determination were not carried out.

Ultracentrifugal analysis.

In the analysis of the different virus preparations a study of their sedimentation in centrifugal fields proved to be most enlightening. In these studies two different methods of recording were employed.

The so called separation cell (153) is divided by a perforated membrane into an outer and an inner compartment. After the outer, bottom compartment has been filled with test solution the membrane is covered with a thin filterpaper and finally the inner compartment filled as well. This simple device prevents convection between the inner and outer compartment when the centrifuge is stopped, and the contents of these can be sampled and titrated separately. By this means it is possible to study the sedimentation of a biologically active substance even if its concentration is far below the limits of direct observation.

For optical registration Lamm's scale method (94) was used, by which the refractive index and consequently the concentration gradients are directly recorded.

A. *Biological recording.*

By computing the sedimentation velocity from the data obtained by this method, it is usually assumed that the substance sediments with a sharp boundary. The position of the boundary can be calculated from the distribution of material on the two compartments of the cell.

In our case the titration method has a percentage error that is independent of the actual concentration of virus. Thus the absolute error will be smaller, the lower the concentration. This means that the sedimentation boundary can be located with greater accuracy the nearer it comes to the dividing membrane.

In preliminary experiments the most suitable conditions for accomplishing this purpose were studied and found to be centrifugation in a centrifugal field of 18 800 g. for 90 minutes at 0° C.

The final tests were made on material of different degrees of purity, from crude suspensions to highly purified preparations. Two series of experiments were carried out. In the first series the test solution was spun for 45 and 90 minutes. The contents of the two compartments were sampled and titrated in mice together with a control. The other series was identical except for the centrifugation times. Sampling was made after 30, 60, and 90 minutes. The titration results are summarized in table XX.

It will be remembered that the standard error of the difference $\log A - \log A_0$ between a test sample and the control amounts to 0.292, which is more than the expected difference itself in the case of runs of 30 minutes. It is, therefore, obvious that incidental variation might have a very great influence upon the relative titer values, and that the effect must be more pronounced the shorter the centrifugation time. In order to obtain more reliable figures the mean differences and their standard errors were calculated for the whole series of experiments. On this basis the relative concentrations were computed with the control as unit. These figures are found in the two bottom rows of table XX. Likewise the mean values of the other centrifugation data were calculated: the meniscus position, the volume of the inner compartment, and the integral of the time-velocity curve.

The titer values of the outer compartment are obviously too uncertain to serve as a basis for any calculations. Furthermore one will

Table XX.

Centrifugation in the separation cell. Titration data.

Run	Degree of purity	Inner compartment				Outer compartment				Control
		30′	45′	60′	90′	30′	45′	60′	90′	
1	crude		6.82		6.27		6.75		7.10	7.14
2	crude		8.47		7.70		8.51		8.65	8.39
3	crude		7.94		6.92		8.66		7.93	8.11
4	medium		7.33		6.66		8.17		8.44	8.43
5	medium		7.46		7.07		8.25		8.59	8.12
6	medium		7.52		7.02		8.29		8.43	8.28
7	medium		8.94		7.69		10.06		9.78	8.91
8	medium		8.94		7.87		9.47		9.69	9.41
9	high	8.31		8.06	7.41	8.68		8.32	8.60	8.11
10	high	9.02		8.21	8.24	9.56		9.56	9.48	9.24
11	crude	7.14		7.04	5.64	7.36		7.58	7.32	7.71
12	high	6.89		6.86	6.16	7.30		7.58	7.51	7.23
medium rel. conc.		0.59	0.38	0.28	0.063	1.42	1.48	1.54	1.60	
limits.....		0.74	0.48	0.35	0.075	2.55	2.74	2.90	2.76	
		0.46	0.30	0.22	0.049	0.49	0.68	0.84	1.30	

Preparations 1 and 11 were crude suspensions, 2 and 3 were digested with pancreatic juice, 4, 5, 6, 7, and 8 were purified by differential centrifugation, 9 and 12 were purified by ammonium sulphate precipitation and centrifugation, and 10 by ether-treatment and centrifugation.

find, on calculating the total virus content of the cell, a definite deficit in comparison with the control. This is a regular occurrence in the case of high molecular gel forming substances. Part of the material is adsorbed in the filter paper, and part of the sediment at the bottom of the cell will not be resuspended. This deficiency must, therefore, be localized principally to the outer compartment.

On account of these circumstances it was decided to base the computation of sedimentation velocity exclusively on the decrease of the virus content in the inner compartment, thereby using the mean values described above. The result is illustrated in fig. 8, showing the position of the boundary, centrifugation time on the abscissa, log distance from the axis on the ordinate. The vertical line through each point denotes the standard deviation of titer. To this should be added the variation of meniscus position, cell volume and other similar centrifugal data. This operation, being extremely compli-

cated, was omitted. It was considered sufficient to remark that the total variation with a certain small amount exceeds the one marked in the figure. For special technical reasons this additional error could be expected to be particularly large for the 45 minutes' value.

Three of the points of the diagram fit in excellently with a straight line through the zero point. The fourth, which happens to be the most uncertain one, the 45 minutes' value, deviates from this line

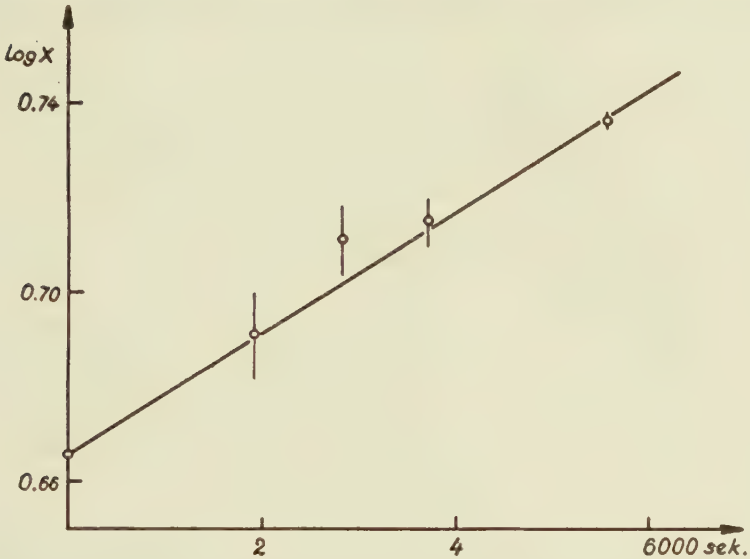

Figure 8. Sedimentation of mouse poliomyelitis virus in the separation cell. Titration data. The vertical line through each point represents the standard deviation of titer.

to an amount of the same order of magnitude as its own variation. It might therefore be safe to assume that the points of the diagram really represent a straight line. This in its turn would mean that the virus is homogeneous with regard to sedimentation. For if the virus split into several distinct active fractions, the course of the line would be curved from the start, concavity towards the abscissa. If on the other hand a single but inhomogeneous active component were present, the line would be straight at first, but as soon as the concentration gradient of the boundary reached the membrane, its course would curve downwards.

The sedimentation constants were calculated for each centrifugation time with the precautions mentioned above (Table XXI).

Table XXI contains also the total virus content of the cell, the control as unit, calculated on the basis of the titer values. The previously mentioned deficit of activity is evident, increasing with time. This indicates a loss of virus with the ever closer packed sediment at the bottom of the cell, from where it is not easily resuspended, rather than through adsorption in the filter paper. It should be noticed, that the 45 min. value falls out of line in this series as well.

Table XXI.

Sedimentation constants and total virus content of the cell on the basis of titer values from table XX.

Centrifugation time	Sedimentation constant	Total virus content
30 min.	151×10^{-13}	1.01
45 »	195×10^{-13}	0.90
60 »	154×10^{-13}	0.90
90 »	151×10^{-13}	0.80
weighted mean	152×10^{-13}	

The weighted mean of all four values is 152×10^{-13}.[1]

B. *Optical recording.*

A few of these experiments were carried out in the oil turbine centrifuge at 21 000 r. p. m., but in the majority of cases an electrically driven "equilibrium" centrifuge was used. The top speed was 18 000 r. p. m., corresponding to a field of 18 800 g. All runs were made at 0° C. The data were recorded and the results computed as usual. All figures were corrected to + 20° C.

In a series of preliminary runs crude suspensions of normal as well as infected brains were studied. The material proved to be very inhomogeneous. Sometimes more distinct components were observed, but these observations usually could not be reproduced and confirmed, the reason for which might have been that the system was very sensitive to small alterations of the salt concentration.

[1] In the preliminary communication the constant was calculated to $160—170 \cdot 10^{-13}$. This estimation was based on individual values from part of the runs now reported and must be considered less accurate than the figure given above.

The first more definite result was obtained with preparation II (experiment 19). In repeated experiments reproducible sedimentation diagrams were recorded. Fig. 9 shows one of these. The heavy line represents the concentration gradients actually recorded. The fine dotted line is an attempt to resolve the diagram into its constitutional elements. The material thus seemed to contain four components, three of which were homogeneous, the fourth and major component, however, being largely inhomogeneous. The sedimentation constants

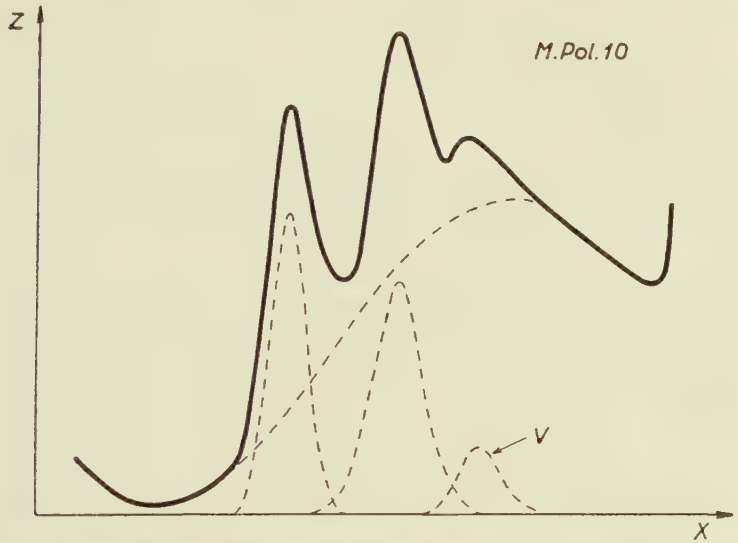

Figure 9. Sedimentation diagram of preparation II. V = virus component. Centrifugal field 32 000 g. Centrifugation time 40 minutes.

of the distinct components were $s_{20} = 81$, 118, and 144×10^{-13}. Of these only the latter is in the range where the virus would be expected. In four experiments the values 144, 151, 147, and 157, averaging 150×10^{-13} were found. On account of the uncertainty in interpretation of the diagram no calculations of the concentrations were attempted.

Preparation III (experiment 20) showed when analysed the presence of two main components. The concentration was, however, too low for accurate observations. Through high speed centrifugation and resuspension the material was concentrated 5 times, after which the diagram in fig. 10 was obtained. Of the three main components one is homogeneous with a sedimentation constant $s_{20} = 151 \times 10^{-13}$,

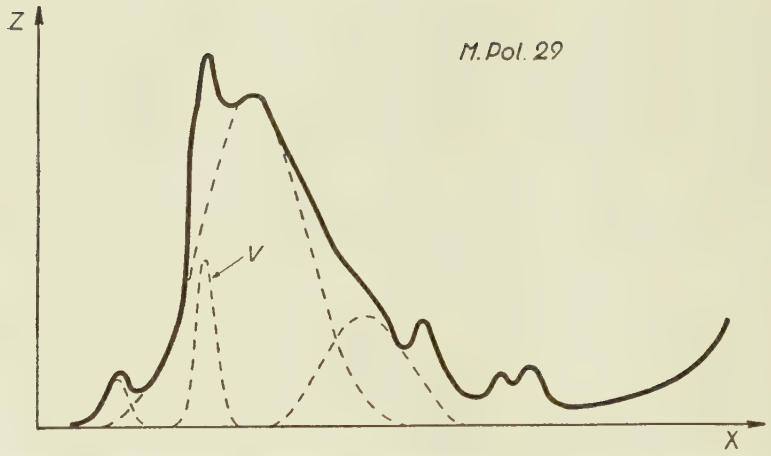

Figure 10. Sedimentation diagram of preparation III. V = virus component. Centrifugal field 32 000 g. Centrifugation time 10 minutes.

the others inhomogeneous. The most distinct of these has the constant 195×10^{-13}, the third is still higher. Fig. 11 demonstrates the sedimentation of preparation V, purified in the same way as preparation III. The homogeneous component has the constant 153×10^{-13}.

Figure 12 contains diagrams obtained with two different preparations. At the bottom are two exposures from the same run of preparation VII (experiment 21), showing three distinct components with sedimentation constants 39, 165, and 232×10^{-13}. The top diagrams are the corresponding exposures of a preparation of normal

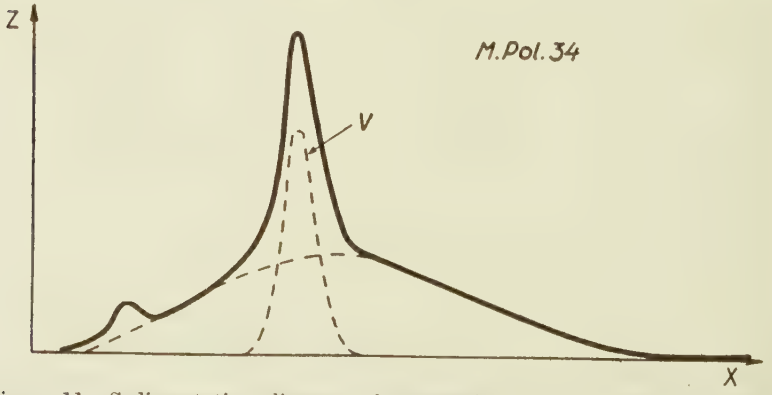

Figure 11. Sedimentation diagram of preparation V. V = virus component. Centrifugal field 32 000 g. Centrifugation time 17 minutes.

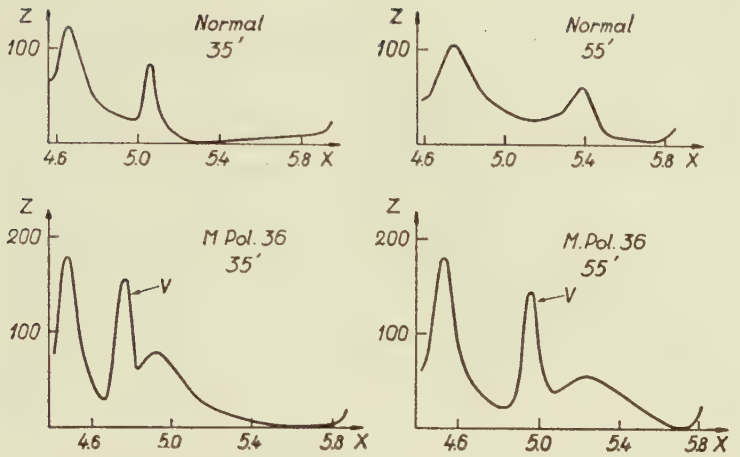

Figure 12. Sedimentation diagrams of purified normal material (top) and virus containing brain (bottom). V = virus component. Centrifugal field 18 800 g. Centrifugation times 35 and 55 minutes.

brains, purified in exactly the same way. Only two components are visible, the constants of which are 40 and 229×10^{-13}. *Thus material from infected and from normal brains had two components in common, while the infected brain alone contained a third and very distinct one, the sedimentation velocity of which was of the same order of magnitude as that of the virus.*

Figure 13 shows the sedimentation of preparation IX (experiment 23). In this case the substance was apparently homogeneous. No

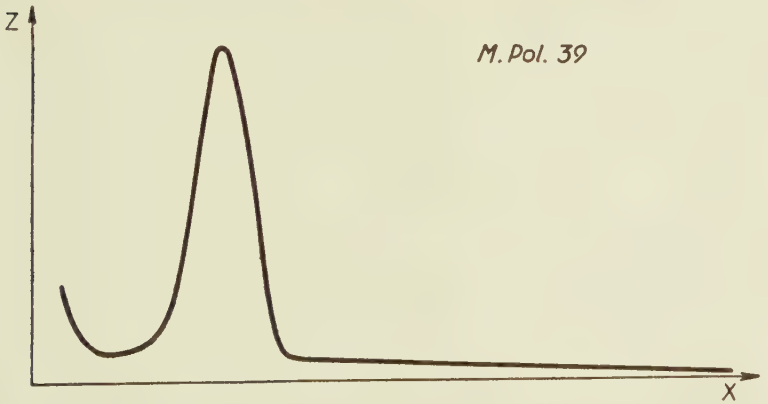

Figure 13. Sedimentation diagram of preparation IX. Homogeneous. Centrifugal field 18 800 g. Centrifugation time 31 minutes.

traces of impurities could be detected. The sedimentation constant was 181×10^{-13}. Unlike the previous centrifugations, in which distilled water was used as medium, this one was carried out in phosphate buffer of ionic strength $\mu = 0.05$. When the experiment was repeated later, using distilled water, a sedimentation constant of 159×10^{-13} was obtained. Experiment 23 was, however, exceptional. In three more cases, when the same purification method was employed, sedimentation diagrams like figure 14 were recorded. The characteristic feature was that the meniscus side of the gradient was normal, whereas the bottom side was disturbed. In later exposures of the same run the inhomogeneity would often have spread completely.

Figure 14. Sedimentation diagram of purified virus showing disturbance of the gradient. Centrifugal field 18 800 g. Centrifugation time 22 minutes.

so that no gradient referable to a disturbing component could be discovered. The preparation would then appear homogeneous. The inhomogeneous substance amounted to from 20 to 50 p. c. of the total contents of solids. For reasons to be discussed later it was assumed that this component consisted largely of the homogeneous substance in aggregated state and with adhering particulate impurities.

In the experiments now described it was found that purified and concentrated material from infected brains contained a substance that was not present in normal brains similarly treated. This substance displayed homogeneity of sedimentation with a sedimentation constant approximately equal to that of the virus as determined by means of biological recording. It was distinguishable in all preparations with a titer above log A = 9.50. When the concentration was calculated from the sedimentation diagrams and compared with the titer of the corresponding preparation the following series was obtained (table XXII).

Table XXII.

Connection between concentration of virus protein and titer.

In the calculation of concentration the refractive index was assumed arbitrarily to be that of proteins.

Preparation	Concentration of virus protein p. c.	Titer	MID per mg
IV.....	Not distinguishable	8.81	—
II	Uncertain, below 0.010 p. c.	9.30	More than 2.0×10^{10}
VI.....	0.010 »	9.70	5.0×10^{10}
III	0.015 »	10.21	10.7
XXI.....	0.021 »	10.25	8.5
V.....	0.025 »	10.24	6.9
VII.....	0.075 »	10.34	3.0

There is an unmistakeable correspondence between the two entities. The last column contains the number of MID per mg of substance. These figures show a variation that might appear systematic but is probably merely incidental. Firstly it is of the same order of magnitude as the standard error of titration, secondly there is apparently no connection whatsoever with purification methods employed or degree of purity of the preparations. It might therefore be assumed that the activity is proportional to the concentration of the component in question.

All those circumstances taken together strongly suggest that *the substance now discussed represents the virus protein itself.*

So far no attempts have been made to analyze it chemically, as the only apparently pure preparation obtained (prep. IX), amounting to a total of 0.6 mg, was used for determination of the diffusion constant. During the sedimentation studies, however, some observations of a certain interest from a physico-chemical point of view have been made.

In 7 runs on 4 different, comparatively impure preparations (less than 15 p. c. virus), the sedimentation constant was calculated to $150 \pm 4.3 \times 10^{-13}$. 6 highly purified preparations (25—100 p. c. virus) gave in 7 runs an average of $162 \pm 6.1 \times 10^{-13}$. The difference, 12 ± 7.5 is not statistically significant but indicates strongly that aggregation occurs during the purification process. We will return later to this question.

As already mentioned, preparation IX had a sedimentation constant of 181×10^{-13} in dilute buffer solution as compared with 159×10^{-13} in distilled water. The difference is too big to be incidental. The signification of this fact is not immediately obvious. It might be interpreted as a salt effect on the state of hydration, similar to that studied by Bernal and Fankuchen (14) in tobacco mosaic virus (cf. page 147).

The yield of virus was surprisingly uniform. In 7 experiments it was 0.43, 0.83, 0.50, 1.67, 0.55, 0.21, and 0.50, averaging 0.60 γ per mouse brain and representing from 20 to 50 p. c. of the original virus content. This is in close agreement with the biological experience. As already mentioned, the virus multiplies in the CNS until a certain critical level is attained. This level seems to be never exceeded but always reached, and appears to be a characteristic of the strain of virus.

II. Human neurovirus.

The scarcity of monkeys made it impossible to control in detail the applicability on the human virus of the experience gained in the purification experiments of the preceding section. It was therefore decided to apply the final method of purification to human material and to study the preparations in the ultracentrifuge, in order to ascertain, whether or not any components comparable to the mouse virus protein could be obtained.

The preparation of extracts, treatment with ether, precipitation with ammonium sulphate (40 p. c. saturation), and ultrafiltration was carried out as previously described. The concentrate was fractionated twice in the Beam's centrifuge (run up-and-down to 16 000 r. p. m. and 70 minutes at 27 000 r. p. m.). The final sediment was resuspended in 1 ml of distilled water.

In a preliminary experiment a normal control from two brains and spinal cords was prepared. When analysed in the ultracentrifuge it proved to contain one single, comparatively homogeneous component, the sedimentation constant of which was about 60×10^{-13}.

Next a material consisting of brains and spinal cords from two cases of poliomyelitis was purified (preparation XXX). In the ultracentrifuge the normal component was observed and besides that traces of a faster sedimenting substance. The sedimentation constant of the latter was probably below 200×10^{-13} but could not be

determined with any degree of accuracy on account of the low concentration. 0.05 ml of this material was inoculated intracerebrally to a Cynomolgus monkey, MC 1. The temperature chart of this animal is shown in fig. 15. A rise in temperature occured on the 9th day after inoculation, followed by a collapse-like fall on the 12th day. On the 11th day the first motor symptoms were evident, consisting of paresis of the left arm. On the 12th day complete paralysis of the left arm and the right leg, prostration. The animal was sacrificed. No gross lesions were found at autopsy. Sections of pons and spinal cord showed typical lesions (Plate II). It was thus obvious that

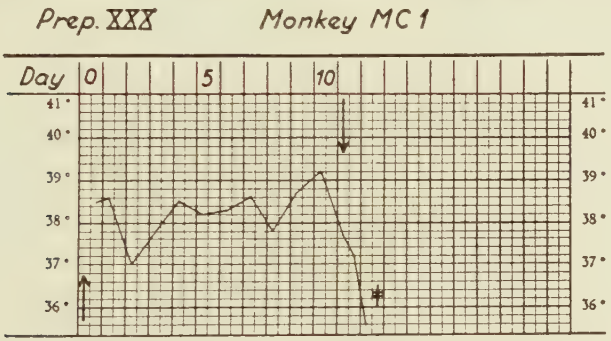

Figure 15. Temperature chart of monkey *MC 1*. † time of inoculation. ↓ onset of paralysis. ∓ animal sacrificed.

preparation XXX contained virus, although the concentration was too low for optical recording. Titration of the activity was unfortunately out of question.

Finally a pool of material from 11 cases was purified. This time the pedunculi cerebri et cerebelli were cut through and cerebrum and cerebellum discarded. The remainder — midbrain, pons and medulla oblongata et spinalis — amounted to about 5 p. c. of the total CNS, while its virus content can be estimated to about 90 p. c. of the total. By this operation, therefore, a substantial simplification of the purification procedure should be attained without any considerable loss of virus. The final preparation (XLVII) was faintly yellowish red in colour like the two preceding ones, but appeared in contrast to these to be inhomogeneous. On high speed centrifugation a small amount of an oily sediment was deposited, as in the earlier preparations, but in addition a minute, clear, gelatinous pellet was

present, of the same general appearance as the preparations of the mouse virus. The sedimentation diagram showed the presence of the normal component, and furthermore two additional homogeneous components in low concentration were distinguishable (fig. 16). The sedimentation constants of these were about 100×10^{-13} and 150×10^{-13}. The latter value is of the same order of magnitude as that of

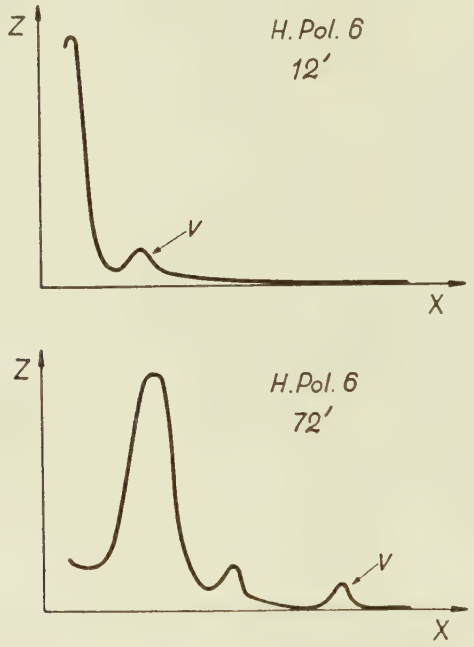

Figure 16. Sedimentation diagram of preparation XLVII, human poliomyelitis. V = virus component. Centrifugal field 18 800 g. Centrifugation times 12 and 72 minutes.

the mouse virus. Activity tests on preparation XLVII could not be performed.

The results of these experiments on the human neurovirus were less conclusive than those on the mouse virus. Firstly activity tests could not be carried out to the desirable extent, secondly insufficient amounts of material were prepared. Thus it has not been ascertained, whether one or both of the additional components in the last experiment are not normal constituents of the spinal cord, as the control was not directly comparable to this preparation. It might, however, be assumed that the component, which was traced in preparation

XXX but absent in the control, is identical with the fastest sedimenting substance in preparation XLVII, and that the presence of this substance is referable to the fact that the preparations were derived from infected material. As one of the preparations was shown to be active, it must be considered plausible to assume that the activity is connected with the component in question. At least a fraction of the virus, therefore, seems to have a sedimentation constant of about 150×10^{-13}, i.e. within the limits of the experiment the same as that of the mouse virus. The question whether or not the middle component of preparation XLVII has any connection with the activity can not be settled at present.

CHAPTER VI.

Purification of intestinal virus.

With the exception of the experiments by the present writer already mentioned (46), no attempts to purify virus from stools or intestinal contents have been reported.

The general procedure adopted in the following experiments was a direct application of the experience from the purification of neurovirus. It was in detail as follows.

The material was suspended in a suitable volume of 0.3 p. c. saline solution and shaken with glass beads in a shaking machine for 2—4 hours. Further diluent was then added and the mass extracted under mechanical stirring for 8 hours. It was then left to settle in the cold room over night. The volume of fluid added was chosen so as to let the sediment amount to 10—15 p. c. of the total. The supernatant was siphoned off and passed twice through the separator. The clarified extract was mixed with 2/3 by volume of saturated ammonium sulphate solution and 1/10 by volume of ether, thoroughly shaken and left to settle in a separatory flask. After 4—8 hours the clear bottom layer was drawn off and the top layer resuspended in 3 times its volume of distilled water with an excess of ether. After 12 hours in the cold room the extract was collected and the top layer extracted further 2 or 3 times. The pooled extracts were clarified in the separator and concentrated through ultrafiltration. The concentrate, usually dark brown, turbid, and viscous, was run in the preparatory centrifuge at 27 000 r. p. m. for 90 minutes. The supernatant was discarded and the sediment allowed to swell in distilled water for at least 2 hours. It was then resuspended to the original volume in distilled water and run up-and-down to 16 000 r. p. m. The second supernatant was further fractionated in the centrifuge, alternatingly at 27 000 r. p. m. for 70 minutes and up-and-down to 16 000 r. p. m.

Most of the following work was done on material of human origin. In order to avoid unnecessary reiterations these experiments will, therefore, be described first.

I. Experiments on human stools.

Differential centrifugation.

A pool of 6 samples from cases of poliomyelitis was prepared as described above. The purified extract was brown in colour, almost clear but with a strong Tyndall cone. On centrifugation at 27 000 r. p. m. for 70 minutes a brown translucent gelatinous pellet was deposited. This pellet was stratified in structure, probably an indication of inhomogeneity. It was soluble in distilled water, more easily after swelling in a small volume of water for 1 or 2 hours. When analysed in the ultracentrifuge it showed the presence of three main components with sedimentation constants 147, 210, and 276×10^{-13}. The solution = preparation *c* was subjected to further purification as follows.

The volume was made up with distilled water to 100 ml, giving a very faint colour but a distinct Tyndall phenomenon. The solution was run up-and-down to 22 000 r. p. m. The supernatant was collected, the sediment redissolved in 100 ml of distilled water and again run up-and-down to 22 000 r. p. m. The procedure was repeated until the supernatant was free of slower sedimenting material, in all 5 times. The final sediment was redissolved in distilled water = fraction γ.

The pooled supernatants were concentrated through ultrafiltration to a volume of 100 ml = preparation *b*, and fractionated in the centrifuge in principally the same way as before, only being run up-and-down to 27 000 r. p. m. instead of 22 000. More than 20 runs were necessary to make the separation complete. The final sediment was redissolved in distilled water = fraction β.

The second pooled supernatants were again concentrated through ultrafiltration to a volume of 100 ml = preparation *a*. The solution was centrifuged at 27 000 r. p. m. for 25 minutes. As a complete sedimentation of the material seemed to occur no further fractionation was attempted. The sediment was redissolved in distilled water = fraction α. In each step of this fractionation procedure the centrifugal effect applied was approximately twice that of the preceding one.

Thus preparation *c* contained fractions α, β, and γ, preparation *b* fractions α and β, and preparation *a* only fraction α. Of each solution 0.5 ml amounts had been inoculated intracerebrally into monkeys. All three animals came down with typical poliomyelitis. Their temperature charts are shown in fig. 17 and micrographs of sections from the CNS in plate II.

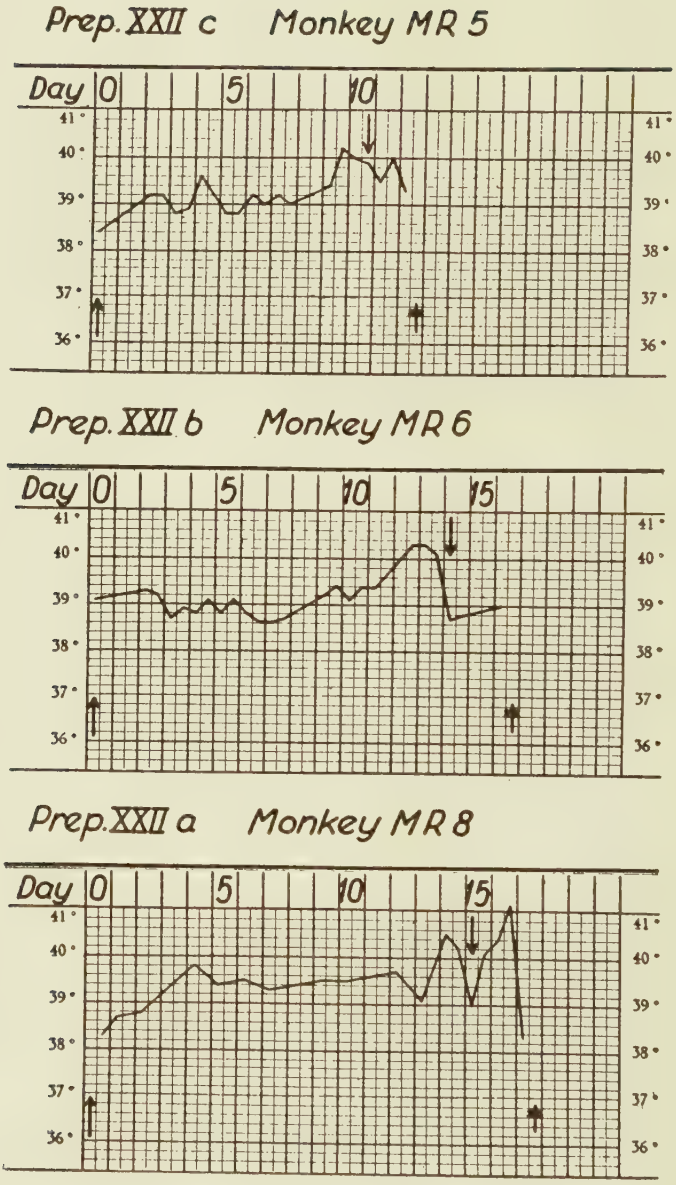

Figure 17. Temperature charts of monkeys *MR 5, 6,* and *8.* ↑ time of inoculation. ↓ onset of paralysis. ‡ animals sacrificed.

Rhesus monkey MR 5, preparation *c*: Rise in temperature on the 9th day. Paresis of left arm on the 10th day. On the 12th day temperature was back to normal, the paresis slightly more pronounced but no further motor symptoms had appeared.

Rhesus monkey MR 6, preparation *b*: Rise in temperature on the 11th day, reaching a peak on the 12th and 13th day, back to normal on the 14th day, when paresis of the hind quarter was observed. On the 15th day complete paralysis of legs, paresis of left arm. General condition good. 16th day no further development of symptoms.

Rhesus monkey MR 8, preparation *a*: Rise in temperature on the 14th day. Remission to normal on the morning of the 15th day, paresis of right leg. New rise in temperature in the afternoon, culminating on the following evening, when complete paralysis of hind quarter and paresis of left arm were observed. On the morning of the 17th day temperature below normal. Animal prostrated.

Preparation *c* had a total volume of 12 ml, *b* and *a* 100 ml, as already mentioned. Monkey MR 5 therefore received an amount of fractions α and β about ten times that administered to MR 6. The amounts of fraction α, given to MR 6 and MR 8 were approximately equal. The fecal specimens were collected on October 14. Preparation *c* was ready for inoculation on November 8, preparation *b* on November 29, and preparation *a* not until January 28, 3 1/2 months after the start of the experiment.

A gradual increase of the incubation period was evident. On the other hand the clinical course of the experimental disease was gradually aggravated. When all circumstances are taken into consideration, the assumption that *the activity had principally followed fraction* α seems best to meet with the facts.

The three fractions were very similar in appearance. They all formed brown, translucent pellets. The colour of γ was deep brown, α and β were of a somewhat lighter shade. α seemed to be more easily soluble than the others. The solutions were clear with a pronounced Tyndall phenomenon. When observed in the dark field (magnification 600 ×) γ contained a mass of particles, just about visible, often in larger aggregates, whereas no structures whatsoever could be detected in solutions of α and β.

The solutions (in distilled water) were analysed in the ultracentrifuge. Each fraction consisted of one single component. The sedimentation constants were: $\alpha = 42$, $\beta = 79$, and $\gamma = 309 \times 10^{-13}$. The diagrams of α and β indicated that their sedimentation velocity was dependent upon their concentration. In a number of experiments

this dependence was studied. The result is summarized in the diagram in fig. 18, sedimentation constant on the ordinate, and concentration on the abscissa. The two curves, not referring to absolute concentrations, are comparable only concerning their general shape. Dry weight determinations were not carried out and calculations of the concentration from the sedimentation diagrams were at least uncertain, since the nature of the substances and their refractive

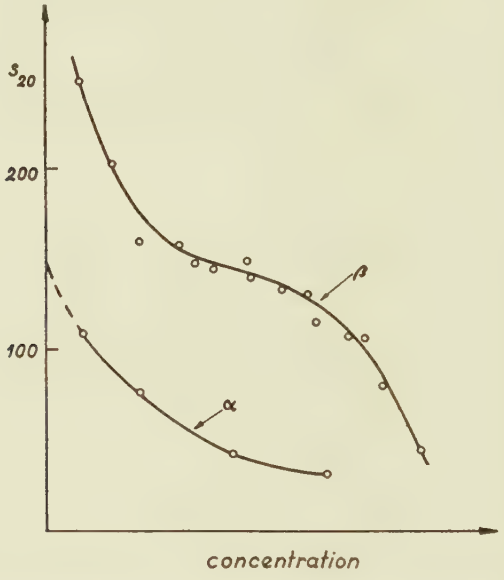

Figure 18. Sedimentation velocity-concentration curves of the α- and β-component of human stools.

indices were unknown. From fig. 18 it is evident, that under certain conditions separation of a mixture through centrifugation might be impossible, and that the two components might sometimes be made to change places in the sedimentation diagram by adjustment of the concentration. If, however, the mixture is diluted above a certain limit the difference in the rates of sedimentation can always be brought to an amount that makes separation although laborious yet possible. Hence the dilution to 100 ml in the above separation procedure.

For many substances showing variation of sedimentation velocity with concentration the following formula is approximately valid

$$s_c = s_0 \cdot \frac{1}{1 + kc}$$

where s_c and s_0 denote the sedimentation constants at the concentrations c and 0, and k is a constant. In those cases s plotted against c. s will give a straight line. The use of the latter method of plotting often simplifies extrapolation of the value of s_0. As the α component seemed to follow this formula, the ultimate value of its sedimentation constant for the concentration 0 could be estimated to about 165×10^{-13}. On the β component on the other hand the formula was not applicable, and in estimating the upper limit of the sedimentation constant great caution was necessary. The value was probably above 300×10^{-13}.

Effect of sodium chloride.

The presence of small amounts of salt had a very marked effect on the sedimentation. The γ component seemed to be practically insoluble even in 0.01 m sodium chloride solution and could easily be disposed of through centrifugation at 3000 r. p. m. β appeared more homogeneous in salt solutions than in distilled water (fig. 19). The rate of sedimentation was higher and less dependent upon the concentration. The upper limit appeared to be about 280×10^{-13}. The salt effect on the α component was somewhat less pronounced. The upper limit of s_{20} was probably about 220×10^{-13}, and values below 100×10^{-13} were exceptional.

It was observed that the pellets deposited at high speed centrifugation from saline solutions were smaller in volume than those obtained from the same preparation in distilled water. They were also less readily soluble. It was probable, therefore, that the salt effect consisted mainly of a dehydration of the substances. As a hint that very high degrees of hydration sometimes were attained, the occurrence of sedimentation velocity curves of the type shown in fig. 20 was considered. The gradually decreasing rate of sedimentation was very conspicuous. Such curves were observed only when distilled water was used as solvent, and most often only the β component was involved. On some occasions though, as in the example quoted, pure α solutions showed this phenomenon as well. This is the type of curve one would expect in a gel, where most of

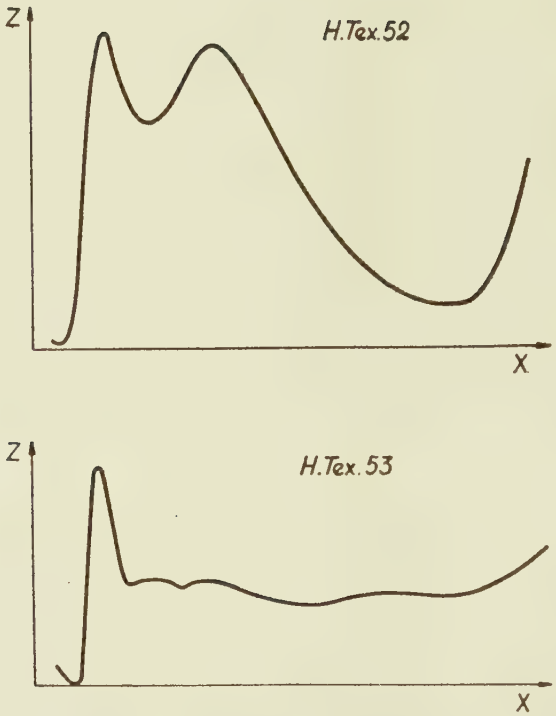

Figure 19. Sedimentation diagram of the same preparation in 0.05 m sedium chloride (top) and distilled water (bottom). The β-component inhomogeneous in absence of salt. Centrifugal field 18 800 g. Centrifugation times 41 and 120 minutes.

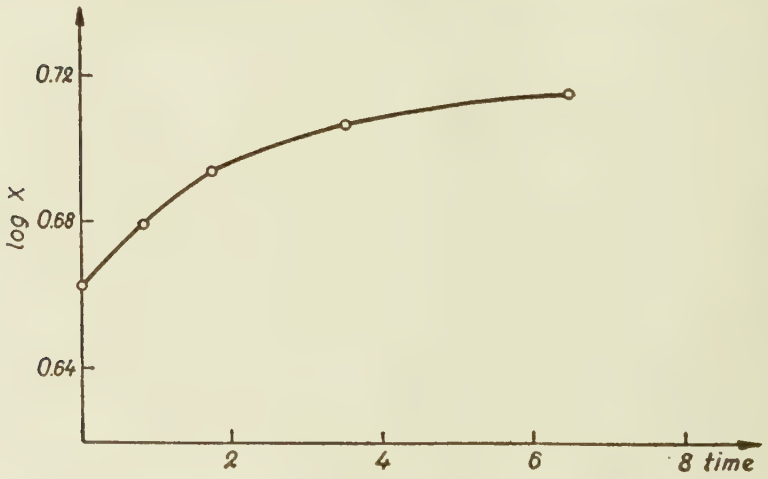

Figure 20. Gellike sedimentation of the α-component in distilled water. Rate of sedimentation gradually decreasing.

the water is fixed. The sedimentation would, in fact, be expected to meet with an increasing resistance, as the force necessary for dehydration increases with the amount of water pressed out of the gel. The sedimentation of gels, however, is not yet sufficiently studied, so the interpretation of the phenomenon now described remains undecided.

In solutions, containing more than one component, conditions were sometimes very complicated. As an illustration fig. 21 shows

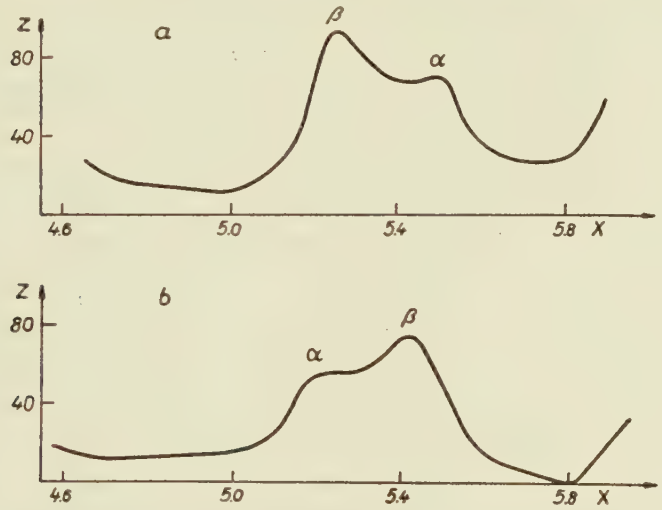

Figure 21. Sedimentation diagrams of the same preparation in distilled water (top) and 0.05 m sodium chloride (bottom). Centrifugal field 18 800 g. Centrifugation times 100 and 72 minutes.

two sedimentation diagrams of the same preparation. In distilled water the α component sediments faster, in 0.3 p. c. NaCl solution the β component. Still other irregularities seemed to be caused by the presence of further impurities. On the whole it was found impossible to identify the components of a preparation by sedimentation analysis alone, and the hope that this procedure might be available for diagnostic purposes had to be abandoned.

The presence of small amounts of sodium chloride seemed, however, in a way to stabilize the conditions of the solution and make the state of the system more well-defined. It should therefore be preferable to carry out the differential centrifugations using a saline solution as solvent. The standard diluent, 0.3 p. c. NaCl solution, was tried

for this purpose and found to yield quicker results. A complete separation of α and β was usually attained after 5 or 6 fractionations.

Solutions of the pure α component, obtained according to this method, gave sedimentation diagrams like fig. 22 showing a disturbance of the sedimentation boundary of the same type as that observed in neurovirus (cf. fig. 14). This disturbance could not be eliminated by differential centrifugation regardless of the number of fractionations applied to the material.

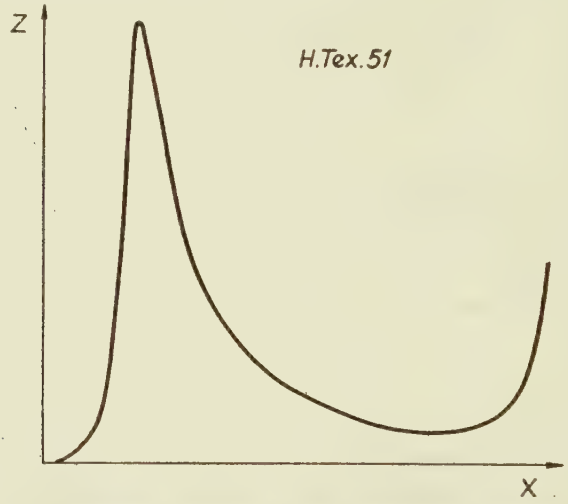

Figure 22. Sedimentation diagram of the α-component showing disturbance of the gradient. Centrifugal field 18 800 g. Centrifugation time 41 minutes.

Treatment with dilute ammonium sulphate solutions.

In a series of experiments mixtures of α and β had been centrifuged in glycerin solutions. The idea was to use differences in specific gravity for separation purposes. Table XXIII illustrates one such experiment. The constants tabulated are not corrected to the density and viscosity of water. The experiment showed that no measurable divergence of the rates of sedimentation was attained by increasing the density of the solvent. The densities of the two substances must therefore be nearly equal and furthermore well above 1.07, approximate specific gravity of 30 p. c. glycerin solution.

For the same purpose ammonium sulphate solutions were tested. In these experiments it was observed that the sedimentation diagrams of solutions of the α component were modified. Fig. 23 shows the same preparation when sedimenting in a dilute phosphate buffer

Table XXIII.

Sedimentation velocities in glycerin solutions.

Solvent	$s_{20} \times 10^{13}$	
0.3 p. c. NaCl	167	197
» + 10 p. c. glycerin	115	155
» + 20 p. c. »	65	94
» + 30 p. c. »	51	70

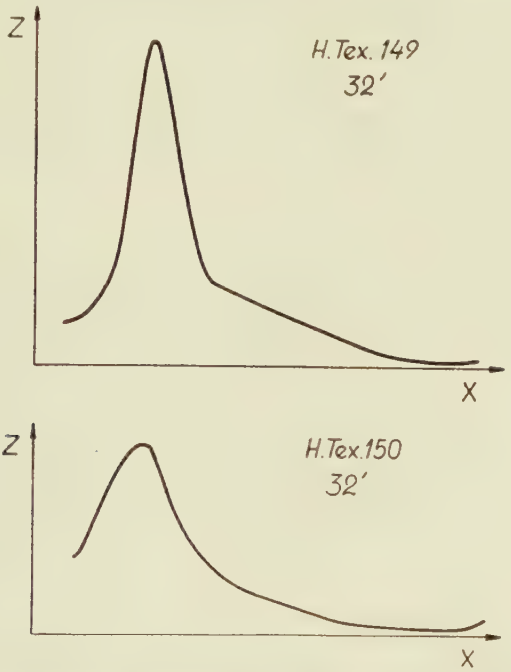

Figure 23. Sedimentation diagrams of the α-component in 0.05 m sodium chloride (top) and 0.2 m ammonium sulphate (bottom). Centrifugal field 18 800 g. Centrifugation times 32 minutes.

solution $\mu = 0.01$ (top) and in 0.2 m ammonium sulphate (bottom). Compared with the control, the bottom diagram showed a higher degree of polydispersity and the disturbance of the gradient was most pronounced on the meniscus side instead of the bottom side. A similar alteration was not observed in 0.2 m NaCl solution or in glycerin medium, neither did it occur in solutions of the β component.

As an attempt to explain this observation it was assumed that the disturbance of the gradient was due to low molecular impurities, firmly adsorbed on the conjectured virus protein. The ammonium sulphate solution should act as an eluent. The free low molecular material would sediment slower, causing a disturbance of the meniscus side of the gradient, while the virus protein, deprived of its protecting coating would be liable to aggregate with polydispersity as consequence.

If this interpretation were correct it should be possible to improve the purity of the material by treatment with ammonium sulphate solutions of a suitable concentration. The elution of the impurities could be expected to be a reversible process, so they should be removed, while the salt concentration was maintained at the most favourable level. To judge from the diagrams, differential centrifugation did not appear to be an ideal method for separation. Instead of that etherization was tried.

To 45 ml of purified α solution 5 ml of saturated ammonium sulphate solution and 10 ml of ether were added, the mixture vigorously shaken in a separatory funnel and left to separate. Beneath the ethereal layer a distinct brown pellicle had formed. The aqueous bottom layer was drawn off and, after removal of the ether, fractionated in the centrifuge. Hereby a further amount of insoluble material could be removed through low speed centrifugation.

The final preparation was almost colourless with a distinct bluish Tyndall cone. When analysed in the ultracentrifuge, it appeared to be more polydisperse than the original preparation, although the previous disturbance of the gradient was not distinguishable (cf. fig. 24).

Occurrence of the α component.

In October 1941 the first series comprising a total of 42 specimens from 6 cases of poliomyelitis was examined. The material was collected at intervals of 2 or 3 days and pooled as indicated in table XXIV. The average time between onset of symptoms and the collection of the specimens included in the different pools varied from 12 to 29 days. Each pool was prepared separately. From all of them the α component could be isolated. The yield was roughly proportionate to the number of specimens included. There was definitely no decrease in amount with increasing length of time between onset of the disease and the date of the specimens.

Table XXIV.

Preparation of stool specimens from cases of poliomyelitis.

Patient	B. K.	O. W.	W. B.	I. B.	I. M.	B. W.	Pool No.	Average time after onset
Onset of disease	26. IX	1. X	1. X	2. X	3. X	8. X		
Specimen collected:								
14. X	+	+	+	+	+	+	XXII	12 days
16. X	+	+	+	+	+	+	XXIII	14 »
18. X	+	+	+	+	+	+	XXIV	16 »
20. X	+	+	+	+	+	+	XXV	18 »
22. X	+	+	+		+	+	XXVI	20 »
24. X	+	+	+	+	+	+	XXVII	22 »
26. X	+	+	+		+	+	XXVIII	24 »
29. X				+			XXIX	29 days
2. XI				+				

In a following series a total of 16 specimens were prepared individually. Of these 6 were collected within one week, 4 in the second week, 2 in the third week, one 3 months, one 4 1/2 months, one 6 months, and one 17 years after onset of the disease. All specimens contained the α component in approximately the same amount, 0.1—1 mg per specimen.

Finally 106 specimens from 27 different patients were examined, partly in pools of 2—6 specimens. The α component was consistently recovered. The yield varied with the amount of solids present in the specimen. It could be estimated to approximately 0.5 mg per 100 g of feces.

A series of controls included 12 specimens from healthy contacts, 2 from obscure lethal cases of polyneuritis, one from a case of acute encephalitis and 19 from miscellaneous cases such as neurosis, polyarthritis, diabetes, vitium cordis etc. 14 specimens were collected in February and March, when no cases of poliomyelitis had occurred within three months before, and the remainder in September during a small epidemic of poliomyelitis. From all of them a substance with the characteristics of the α component was recovered to an amount comparable to that obtained from cases of poliomyelitis.

* * *

The experiments reported in this section had shown that a certain substance, here called the α component, characterized by its precipitability with ammonium sulphate and its behaviour in a centrifugal field, is regularly present in human stools. As far as these experiments go it shows a striking resemblance to the presumed neurovirus protein found in brain and spinal cord from cases of murine as well as human poliomyelitis. Although activity tests could not be performed to the desired extent, suggestive evidence has been presented that in the process of purification most of the original amount of virus, present in active specimens, is to be found in the same fraction as the α component. In such active preparations no further component has been detected that could account for the difference between active and inactive specimens.

II. **Experiments on mouse feces.**

The material was collected as described in chapter II. Batches of about 100 g were prepared at a time. The general procedure of purification outlined in the preceding section was strictly followed.

The preparations thus obtained closely resembled those from human stools. On high speed centrifugation they deposited yellowish brown, gelatinous pellets. The solutions were light brown and clear, with a Tyndall cone. In centrifugal analysis usually only one component was observed. From a physico-chemical point of view this substance was identical with the α component of human stools. The dependence of the rate of sedimentation upon the concentration was of the same order of magnitude. The upper limit of the sedimentation constant was about 160×10^{-13} in distilled water, $190—200 \times 10^{-13}$ in 0.3 p. c. sodium chloride solution.

Sometimes a second component was present but always in comparatively small quantities. It was very sensitive to the presence of salt and therefore more like the γ than the β component.

The sedimentation diagrams of the main component showed a certain disturbance of the bottom side of the gradient, although as a rule to a less extent than the corresponding preparations from human stools. Through treatment with 10 p. c. ammonium sulphate and ether a small amount of impurities could be removed. A sedi-

mentation diagram of a preparation subjected to such additional purification was presented in fig. 24.

The material originated partly from young mice, 5—8 weeks of age, partly from 12—18 months old animals. These two types of preparations showed no differences with regard to physico-chemical properties or yield. The amount of the substance excreted was estimated to about 0.01 mg per mouse and day.

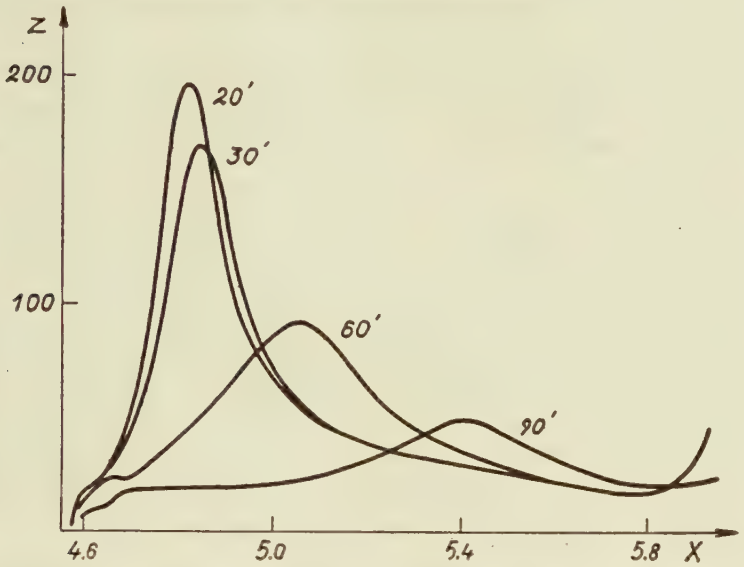

Figure 24. Sedimentation diagrams of mouse intestinal protein, showing polydispersity. Centrifugal field 18 800 g. Centrifugation times 20, 30, 60, and 90 minutes.

Activity tests.

The strains of virus that can be isolated from the intestines of normal mice are usually of a very moderate virulence. In chapter III it was pointed out that titration of less virulent strains met with considerable difficulties. The incubation time method is not applicable. The variation of individual resistance plays an important part in a titration experiment, making the series irregular and the evaluation of the result hazardous.

A phenomenon called interference was reported now and again by workers in the virus field. It was observed that avirulent strains of certain viruses, when inoculated simultaneously with highly

virulent strains of the same virus, modified the course of the ensuing disease or sometimes inhibited it completely. Interference is usually interpreted as a competition for the susceptible cells, this interpretation being based upon the assumption, that a cell once invaded by a virus particle is refractory to superinfection by the same virus. Jungeblut and Sanders (78) have reported experiments with a murine strain of poliomyelitis virus supposed to interfere with simian strains. Similar observations have been made by the present author (unpublished data) on intestinal strains of the mouse poliomyelitis virus and the FA strain. The experiments in question were carried out with impure neurovirus. It was decided to extend this investigation to purified preparations in an attempt to use the degree of interference as a measure of the concentration of the interfering virus.

Experiment 24. In this experiment a purified preparation of feces from young animals was used (preparation XXXVI). It was diluted to 1: 50 and from this standard solution a series of 10fold dilutions was prepared. An extract of infected brains (strain FA) was similarly diluted, starting with 1: 500 (brain wet weight). The dilutions of the feces preparation were mixed with equal volumes of each one of the FA dilutions. Four dilutions of each solution were used making a total of 16 mixtures. Each mixture was inoculated intracerebrally in 0.02 ml amounts to groups of 20 mice. Control titrations of each one of the two solutions were also included. The activity of each mixture was calculated from the incubation time in the usual way and compared with that of the control containing the same concentration of strain FA. The differences $=$ log A—log A_0 are tabulated in table XXV.

Table XXV.

Effect upon the activity of the FA strain by addition of purified intestinal virus.

For explanation of the table see text.

Concentration of FA	Concentration of preparation XXXVI			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
10^{-3}	+ 0.65	+ 0.33	— 0.35	+ 0.07
10^{-4}	+ 1.64	+ 0.02	+ 0.04	+ 0.18
10^{-5}	+ 2.33	+ 1.07	+ 0.16	+ 0.11
10^{-6}	+ 3.26	+ 2.05	+ 1.07	— 0.07

This table is divided into two sections by a heavy line. The values to the right of the line of division are not significantly different

from 0. The mixtures to the left on the other hand have activities significantly higher than the corresponding controls, the increase in activity being approximately proportional to the increase in the concentration of preparation XXXVI. *In this experiment therefore, no interference was distinguishable. The intestinal virus when added to the inoculum had the same effect as would an additional amount of the FA virus itself.*

The control titration of preparation XXXVI gave a 50 p. c. endpoint at approximately 10^{-4}. In the untreated extracts the 50 p. c. point is usually between 10^{-1} and 10^{-2}. The experiment demonstrated, therefore, beyond doubt that virus was present in the purified preparation and that it had been concentrated from the starting material between 100 and 1000 times.

Experiment 25. A second experiment was set up with material originating from old mice (preparation XXXV). This preparation was inactive even if inoculated undiluted. The animals inoculated did not develope immunity as found in a test performed 4 weeks later. The experiment was carried out as follows. A series of dilutions of a FA extract was prepared in the undiluted preparation XXXV and further series in 100 fold dilutions of the same. The activity of each series was determined as usual. The following values of log A were recorded (table XXVI).

Table XXVI.

Effect of purified inactive material upon the activity of the FA strain.

Medium	log A
Prep. XXXV undiluted	9.26
» » diluted 10^{-2}	8.83
» » » 10^{-4}	8.23
» » » 10^{-6}	7.70
Saline solution (control)	7.53

The figures of table XXVI show that the purified preparation, although in itself inactive, could enhance the activity of the FA strain. The effect increased linearly with log concentration.

III. Experiments on further animal species.

Since it had been found that a substance, by the available methods of analysis undistinguishable from the α component of human stools, also seemed to be a normal constituent of mouse feces, it became necessary to include into this study other species of animals as well. The preparation of the specimens was carried out exactly in the same way as before.

Guinea pig.

Minute amounts of substance were obtained, giving a greenish solution, faintly opalescent. In centrifugal analysis only slow sedimenting utterly inhomogeneous material was detected. No distinct components whatsoever were observed.

White rat.

When the preparation was carried out in distilled water comparatively large quantities of slow sedimenting inhomogeneous material were obtained. Most of this was insoluble in dilute sodium chloride solution. No distinct components could be detected in the ultracentrifuge.

Swine.

The intestinal contents of 3 pigs, a total of about 10 liters, were collected at slaughter, and purified as usual. The final preparation consisted of about 1 mg of a homogeneous substance, resembling the α component with regard to sedimentation properties. Before definite conclusions be drawn, however, further studies are necessary.

Wild brown rat (Mus decumanus).

Wild rats were trapped on a garbage dump and caged. Their feces were collected and prepared in the usual way. The purified preparations contained consistently two homogeneous components in large amounts. One of those seemed to be identical with the α component. The other had a lower rate of sedimentation and was only moderately sensitive to dilute salt solution. At repeated fractionation in the centrifuge it seemed to aggregate gradually and could be removed by and by through low speed centrifugation. After reprecipitation with ammonium sulphate (40 p. c. saturation)

the preparations seemed to be free from this component and behaved exactly as the pure α component.

In a number of experiments this substance was tested for biological activity.

Experiment 26. 0.5 ml of the purified material was inoculated intracerebrally to a Rhesus monkey, MR 9. On the 8th day after inoculation rise in temperature of about 1° C. Subsequently irregular temperature. On the 10th day a slight coarse tremor of the limbs was observed. On the 11th day still tremor, no further symptoms. On the 14th day temperature normal, no symptoms distinguishable. Animal sacrificed. At autopsy no gross lesions. Site of inoculation without reactions of any kind. A careful microscopical examination of numerous sections from different levels of spinal cord, oblongata, pons, and cortex did not reveal any lesions.

Experiment 27. 0.1 ml amounts of the same preparation were inoculated intracerebrally to 5 adult brown rats. One animal prostrated on the 6th day. Autopsy revealed brain abscess. The others remained well. The experiment was repeated on 10 young rats, about 4 weeks of age. Between the 10th and 30th day after inoculation 6 animals developed a certain weakness of the hind legs and the back, with staggering walk, the backs slightly hunched and the legs more extended than normal. Part of them were sacrificed and hot passages of brain and spinal cord to rats and mice were made. None of the passage animals developed any symptoms and the mice were not immune to mouse poliomyelitis when tested after 4 weeks. The examination of the diseased animals at autopsy revealed a more or less evident symmetric hydrocephalus, extending over the lateral and third ventricles. On microscopical examination signs of atrophy were found in the vicinity of the ventricles but no inflammatory reactions of any kind in any parts of the CNS. In one further experiment of the same type 30 rats in the age of from 16 days to 4 weeks were inoculated. Again a number of animals came down with the same symptoms. Passage experiments were negative and anatomical findings in all essentials the same as above.

Experiment 28. 0.02 ml amounts of the preparation were inoculated intracerebrally to 20 mice. All animals remained perfectly well. When tested after 5 weeks they were not immune to mouse poliomyelitis.

Although these results were suggestive of some biological effect of the purified material, no transmissible agent could be recovered and no lesions resembling those of poliomyelitis could be provoked.

* * *

The experiments reported in this chapter have revealed that a substance characterized by its insolubility in 40 p. c. saturated ammonium sulphate solution and its sedimentation properties, and referred to as the α component, is regularly present in the feces of

man, white mice, and wild brown rats. A substance of presumably the same kind was also found in intestinal contents from swine, whereas no similar constituent was recovered from guineapigs nor from white rats. Judging from the present limited experience, therefore, this substance is not common to all species of animals.

With regard to sedimentation the α component resembled closely the neurovirus protein. It was, therefore, quite natural that material derived from virus containing specimens seemed to have retained most of the viral activity originally present. When analysed in the ultracentrifuge, however, the active preparations differed in no way from the inactive ones. Thus, no component was detected, which the viral activity could be referred to in particular.

When purified intestinal virus from mice was added to the virulent FA strain and inoculated intracerebrally, no interference phenomenon appeared. The addition of intestinal virus enhanced the effect just as would an additional amount of the encephalitogenic strain itself. The α component from non-infective mouse feces, in itself inactive even when injected in large quantities, had a similar although somewhat less pronounced effect when inoculated together with a virulent strain of virus. In one experiment a 50-fold increase in titer was observed.

As to the mechanism of this activation no conclusive evidence is available. An analogy to the spreading factor of Duran-Reynals (31) was considered. In a few experiments testis extracts were tested with negative result. Furthermore extracts of liver, spleen and kidney were examined. All of these had a slight although significant inhibitory effect, probably due to their content of serum. It is possible that an agent acting as spreading factor in nervous tissue should be expected to possess lipolytic rather than mucolytic properties. So far no experiments to that effect have been performed. The result of experiment 27 on rats gives a hint that the explanation of the phenomenon might be something in line with this suggestion.[1]

At any rate the presence in feces of a substance capable of enhancing the activity of poliomyelitis virus, together with the observation that infected nervous tissue contains an inhibitory factor (chapter V, page 97), explains satisfactorily the ease with which virus can be recovered from feces, and the difficulties to establish the strains in intracerebral passages.

[1] The problem will be reviewed later from another aspect. Cf. page 153.

CHAPTER VII.

Further studies on purified material.

I. Physico-chemical studies.

Diffusion experiments.

From the filtration endpoint determinations the size of the virus of poliomyelitis was estimated to 10—15 mμ, or approximately the same as that of foot and mouth disease. Janssen (75) had found in centrifugation experiments with the latter virus a component with the sedimentation constant $s_{20} = 20 \times 10^{-13}$, interpreted by him as the virus protein.[1]

The sedimentation velocities recorded for the poliomyelitis virus were unexpectedly of a different order of magnitude. It was therefore essential to corroborate these findings by determination of the size and shape of the virus particles, based upon the sedimentation data. To this end diffusion experiments were carried out.

The technique employed was that described by Lamm (94). All experiments were carried out at 0° C and the figures corrected to + 20° C as usual. All preparations examined gave diffusion diagrams more or less of the type to be expected from polydisperse or inhomogeneous material. The constants calculated are, therefore, mean values the extreme limits being about ± 10 p. c. The following values were obtained (table XXVII).

For computation of the molecular weight the specific volume of the substance must be known. Proteins usually have a specific volume of about 0.75. For tobacco mosaic virus it was determined by Eriksson-Quensel and Svedberg to 0.646 (36).[2] This has not been confirmed by later workers. Values of about 0.77 have been recorded

[1] These experiments were carried out at the Institute of Physical Chemistry, Uppsala.

[2] The virus preparation available for these experiments was largely inhomogeneous. The possibility of a partial denaturation must be kept in mind.

Table XXVII.

Diffusion constants.

	$D_{20} \times 10^7$
Prep. IX, murine neurovirus	0.32
» XXVIII, intestinal material from cases of poliomyelitis	0.12
» XXXVI, intestinal virus from mice	0.11
» XXXIV. intestinal material from brown rats	0.11

by Bawden and Pirie (8) and by Stanley (143), and similar figures have been found for tomato bushy stunt (103), potato X (99), and tobacco necrosis virus (122). The plant viruses, therefore, seem to have the specific gravity of the proteins. — The animal viruses are not equally well examined in this respect. The large viruses seem to have a density of between 1.15 and 1.20, while those below 100 mμ tend to approach values of about 1.30 (34). Beard and his collaborators (37) give the figure 1.19 for the specific gravity of equine encephalitis virus, which is of a certain interest, as this virus is neurotropic like that of poliomyelitis.

The ordinary methods for determination of specific gravity, the pycnometric or the floatation methods, were hardly applicable in the present case on account of the scarcity of material. It was therefore decided to calculate an approximate value from the rate of sedimentation in glycerin solutions. By the formula for correction to the density and viscosity of water the specific volume was computed from the sedimentation constants of table XXIII. Assuming the viscosity of 30 p. c. glycerin $\eta = 2.5$ and the specific gravity $\rho = 1.073$ the value $V = 0.76$ is obtained, corresponding to a specific gravity of 1.32.

This value refers to the α component of human stools. Taken by itself it is obviously of little significance. The rate of sedimentation has proved to be dependent upon the concentration, and it can not be regarded as self-evident, that this relation should be independent of the medium. Furthermore it is not known whether or not dissociation or aggregation of the substance might occur in glycerin solutions. With these reservations it tends to show, however, that the α component is protein in nature, i. e. in the same direction as the rest of the evidence. It must therefore be regarded as very plausible.

Thus in the following calculations the specific volume was assumed to $V = 0.76$ for neurovirus and the intestinal components alike.

The sedimentation constant of the pure and presumably dehydrated neurovirus protein was found to be 181×10^{-13}. On the basis of this value and the diffusion constant 0.32×10^{-7} the molecular weight was calculated to 57×10^6. Computing the axial ratio of the molecule in the ordinary way one arrives at the value 38: 1. The formula for this computation is developed for the case of ellipsoidal and non-hydrated molecules. It is hardly probable that molecules of the shape indicated by the above axial ratio should be ellipsoidal. It is more plausible that the particles are filamentous or composed of shorter elements associated end to end, in which case the shape would be closer to that of a cylinder. The theory for sedimentation of cylindrical particles has not been developed. When the axial ratio is large as in this case, one might assume, however, that ellipsoidal and cylindrical particles of the same mass and the same length would have approximately the same coefficient of molar friction. On this assumption the dimensions of the particles were calculated to about 12.5×580 mμ.

Under the same conditions, using the sedimentation constant 220×10^{-13} and the diffusion constant 0.11×10^{-7}, the molecular weight of the intestinal component was found to be 200×10^6, the axial ratio about 145: 1, and the size of the particles 12.2×2150 mμ.

Stream double refraction.

As an attempt to check up on the above figures a specimen of human intestinal material was examined by Snellman for stream double refraction with the technique described by him (139).[1] The solution showed no spontaneous birefringence but already at very low velocity gradients stream double refraction appeared. As the apparatus was not constructed for such low velocities exact measurements could not be made. The result was, however, indicative of particle lengths in the range of 5—6000 Ångström units. Furthermore it was evident that the material was rather inhomogeneous with regard to particle size.

It should be remarked, that spontaneous appearance of a bire-

[1] These experiments were carried out at the Institute of Physical Chemistry, Uppsala.

fringent bottom layer, as happens in sufficiently pure and concentrated solutions of tobacco mosaic virus, was not yet observed in solutions of neurovirus or intestinal material; it may be that neither the degree of purity nor the concentration were suitable for the occurrence of this phenomenon.

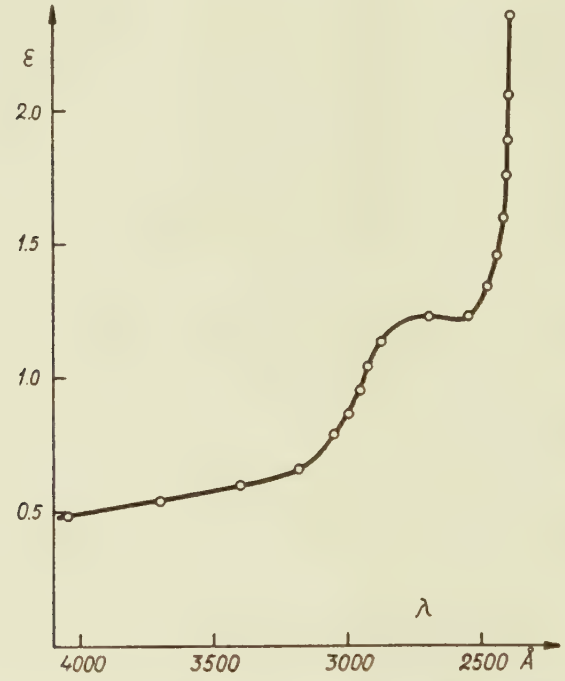

Figure 25. Ultraviolet absorption of purified human intestinal protein.

Ultraviolet absorption.

In these experiments a Judd Lewis spectrophotometer was used. As already mentioned the preparations of intestinal material showed a strong Tyndall phenomenon. In a number of experiments it was found, as could be expected, that the solutions showed a pronounced diffuse absorption in the ultraviolet, increasing towards shorter wave lengths. When it was attempted to eliminate this disturbance by dilution it was found that the sensitivity of the instrument was insufficient for recording the specific absorption. Finally, however, a preparation was obtained from normal human stools, which was

practically colourless and with a comparatively faint Tyndall cone (preparation XLVI). When examined in the spectrograph it gave the absorption diagram shown in fig. 25. This diagram is far from ideal. There is a very pronounced diffuse absorption, making the evaluation of the specific absorption difficult. Chances are that it should be interpreted as a normal protein spectrum rather than that of a nucleoprotein, as the maximum between 2600 and 2700 Ångström might be a false one, provoked by the Tyndall absorption. For definite conclusions examination with a more suitable equipment is necessary.

On the same preparation nitrogen and phosphorus determinations were performed. It was found to contain 16 p. c. nitrogen and 0.6 p. c. phosphorus. As a total of but 2 mg of substance was available, duplicate determinations were out of question. The error, therefore, might amount to $\pm$ 10 p. c. At any rate the figures suggest that the substance be a nucleoprotein with approximately the same nucleic acid content as the rodshaped plant viruses.

II. **Serological experiments.**

Rabbits were immunized by intravenous injections of purified intestinal protein at 5 days' intervals. They each received a total of about 15 mg protein in saline solution, administered in 5 injections. Samples of blood were drawn through heart puncture before the immunization and one week after the last injection. The serums were centrifuged in an angle centrifuge at 3000 r. p. m. for 1/2 hour, frozen and stored without preservatives at - 16° C until used. Three rabbits were immunized, one with protein from cases of human poliomyelitis, another with a similar preparation from young mice, the third one with protein from brown rats.

Complement fixation tests were carried out in the usual way. A purified intestinal protein, the same that had been used for immunization served as antigen. When this was titrated in decreasing amounts against a constant quantity of complement and a constant concentration of normal serum on the one hand, hyperimmune serum on the other, a very substantial non-specific complement fixation was observed. The range between specific and non-specific fixation was too narrow to allow any clearcut results of a titration

of serum. The experiments were repeated with newly prepared proteins with the same results.

Precipitin reactions were carried out as follows. 0.5 ml amounts of a suitable dilution of serum, usually 1: 10, were mixed with an equal volume of antigen solutions of decreasing concentrations, kept at + 50° C for 1/2 hour and further over night in the ice box. Readings were made with a hand lens magnifying 5 times.

With normal serum visible reactions were never obtained. With hyperimmune serum and the homologous antigen a thin, loose flocculation appeared after about 1/2 hour at 50° C. The precipitate was easily broken up at gentle shaking, after which reformation required another 1/2 hour. It hardly settled in the ice box. The reaction was visible in antigen concentrations down to about 1:200 000. The flocculation was decidedly of the type usually associated with filamentous antigens (H-type).

Each serum gave positive reaction with all of the three antigens. No significant differences in titer were observed, but the quantities of precipitate differed, indicating possible antigenic differences. Judged from this basis the proteins from human and mouse feces appeared identical while the protein from rats differed. Absorption experiments were started but gave no conclusive results.

The anti-human serum was tested against a number of preparations from normal stools, of different degrees of purity. When purification and concentration were carried far enough, the preparations gave consistently positive reactions. Hereby were, however, three different types of flocculation observed. Most often the one described above occurred. The precipitate formed seemed to be almost colourless even in dark brown solutions. Sometimes another kind of H-flocculation appeared. The precipitate was coarser, settled readily and was usually coloured, leaving a clear and colourless supernatant. On a few occasions finally, the O-type of precipitation was observed. In these cases the solution appeared milky and turbid immediately after mixing of the reagents. The precipitate was white, granular, and settled in compact masses.

On account of these observations it was felt that the serum contained heterologous antibodies as well, probably originating from impurities present in the preparation used for immunization. Attempts were made to remove these antibodies through absorption. It was soon found, however, that the fractions used for ab-

sorption still contained considerable quantities of the homologous antigen. The result of the absorption experiment tended, therefore, to complete extinction of the precipitin reaction. As it would probably be easier to obtain a pure homologous antigen for immunization than pure heterologous fractions for absorption, the attempts to obtain a strictly monospecific serum were abandoned for the time being.

Until now only one experiment with neurovirus has been performed. A dilution of preparation XXXI, human neurovirus, was mixed with antihuman serum. No flocculation was observed. It was, however, questionable whether the concentration of virus protein in this case exceeded the critical level for visible reactions. The question of the serological relationships between neurovirus and the intestinal protein still remains open.

Neutralization experiments with the hyperimmune serum have not yet been carried out.

So far the serological experiments have not yielded any practical results. The description of this work was included, however, to show that the substances studied possess antigenic properties. It is therefore very probable that a continuation and extension of these experiments will provide more substantial evidence concerning the nature of the intestinal proteins, about which at present only vague theories can be presented.

III. Electron optical studies.

A number of purified specimens were studied in the electron microscope at Laboratorium für Übermikroskopie, Siemens und Halske A.-G. in Berlin. The results have been reported elsewhere (152, 48, 49) and will be only briefly summarized in the following pages. A first series of specimens including the numbers 1), 3a), 4), and 5) (see below) were examined by professor A. Tiselius, the remaining specimens by the present writer.

The commercial type of the Siemens und Halske electron microscope was used, and the technique employed differed in no way from the ordinary (127). All preparations were dissolved in distilled water. Series of tenfold dilutions in distilled water were prepared. Of each dilution a small drop was placed on the object carrier and

allowed to dry at room temperature. A density of the dried preparation, suitable for reproduction purposes was usually obtained with solutions containing 10^{-5}—10^{-7} g protein per ml.

The specimens examined were as follows.

1) Murine neurovirus protein (preparation XXI), according to the sedimentation diagram (cf. fig. 14) probably containing a certain amount of impurities adsorbed.

2) Human neurovirus protein (preparation XLVII, cf. page 111)

3) The α component from human stools.

a) from cases of poliomyelitis, infective to monkeys, not subjected to the final purification (page 124). When examined stored in the laboratory for about 1 year (preparation XXVIII).

b) from cases of poliomyelitis, infectivity not tested. Three different stages of purification were included (preparation XLV).

c) from healthy contacts and control cases. Infectivity not tested. Highly purified (preparation XLIV + XLVI).

4) Purified protein from mouse feces.

a) from young mice. Infective (preparation XXXVI).
b) from old mice. Not infective (preparation XXXV).

5) Purified protein from feces of brown rats, according to the sedimentation diagram containing one more component (preparation XXXIV, cf. page 131).

All specimens examined showed the presence of filamentous structures of principally the same appearance (plate III). The fibres seemed to be very flexible and elastic, a conclusion arrived at on the following grounds. It sometimes happens that the carrier film disrupts at radiation, which provokes a certain tension of the remnant flaps, recognizable on the deformation of the circular defects of the film (127). If this be the case, a structure attached to the film must either break loose, disrupt, or stretch. On a number of micrographs the latter condition seemed to prevail (Plate III e). Fibres lying parallel to the direction of tension were thinned out and straightened, those perpendicular to it were broadened and corrugated.

On this account great care must be exercised in estimating the dimensions of the structural elements. For measurement purposes only such micrographs should be chosen as bear no signs of disturbances of the kind just mentioned. This considered, the length of the particles was measured on a number of micrographs. The result

is demonstrated in fig. 26, where the lengths were arranged in classes of 10 mμ class breadth. The piles of the diagram demonstrate the frequency of each class. Only short fibres, less than 500 mμ were measured. The material was not sufficiently large to permit analysis of each preparation separately, so two pools were prepared, one (I) of murine specimens (1, 4a and 4b), the other (II) of human specimens (2, 3a, 3b, and 3c).

The diagrams each show a series of more or less pronounced frequency maxima. Those denoted *b* originate principally from objects

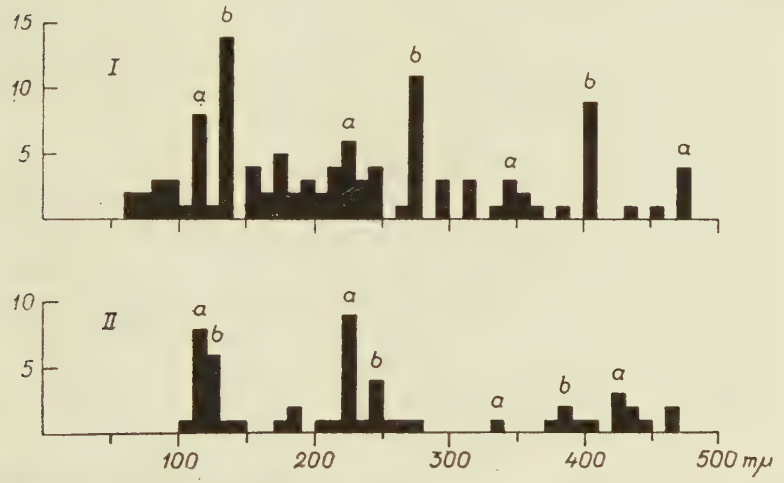

Figure 26. Distribution of particle lengths as measured on electron micrographs. (I) preparations of murine origin. (II) preparations of human origin.

showing tension, while those denoted *a* are from such without signs of disturbance. The frequency distribution is best accounted for by the assumption, that the fibres are composed of a varying number of elementary units about 115 mμ in length, when not subjected to tension or pressure. In this respect there was no significant difference between murine and human specimens.

Measurement of the width of the fibres must be carried out under the same precautions. In that case, however, an extra, uncontrollable cause of variation exists. If two or more fibres are associated side by side, the width of the bundle will appear different when reproduced, depending upon the orientation of the bundle to the carrier film. From plate III d it is obvious that association of that kind

occurs. The arrows in the picture indicate details of the structure, which must be interpreted as the ends of individual fibres of a bundle. On account of this condition statistical evaluation of the fibre width was not attempted. Instead of that measurements were carried out on a number of micrographs showing presumably single fibres not stretched. Thus a mean value of about 15 mμ was obtained, from neurovirus and intestinal proteins, from human and murine specimens alike. As the width of the fibres is near the resolving power of the instrument, the accuracy of the measurement is probably of the order of 2—3 mμ.

Observations on specimen 3b) in different states of purity revealed the following facts. The impure preparation contained beside the filaments two morphologically distinct components. One consisted of rounded particles 50—100 mμ in diameter. The other one was finely dispersed, the elementary particles just about visible, i. e. below 10 mμ. The filamentous phase consisted almost exclusively of single fibres in netlike arrangement. The particulate impurities were anmeshed in this network, while part of the amorphous material seemed to be adsorbed onto it. With increasing degree of purity first the particulate phase disappeared, then gradually the amorphous phase as well. In the purest preparations only the filamentous phase was visible. From comparison with the sedimentation diagrams it was concluded that the large particles corresponded to the γ, the amorphous substance to the β component. Parallel to the degree of purity the arrangement of the fibres changed. An increasing tendency to formation of bundles was obvious. The purest preparations contained hardly any single fibres except as short branches of thicker bundles. One got the impression that the impurities enmeshed in the network prevented association of the fibres mechanically; when these were removed a sort of coagulation of the gel structure ensued.

When the micrographs of the different specimens were compared on the basis of these general observations the following characteristics were found.

The intestinal proteins showed no differences that could not be referred to the actual state of purity. The fibres and their arrangement in active and inactive preparations were identical, and no further elements that could explain the biological activity were observed. Likewise no significant dissimilarities between human, mouse and rat proteins were detected. The sizes of the single fibres

varied considerably; they seemed, however, to be more uniform in the impure specimens. The meshes of the netlike gel structure were of an order of magnitude of about 1 μ.

The micrographs of murine neurovirus showed mainly long filaments, almost exclusively single fibres in a loose network with wide meshes. Now and again impurities appeared to be adsorbed to the fibres, forming bead-string-like structures. The tendency to bundle formation seemed to be less pronounced.

In the human neurovirus preparations a finely dispersed substance predominated. It seemed to be rather uniform with regard to particle size and shape, forming rounded elements of about 10 mμ in diameter. In the bulk of this substance single fibres were embedded, sometimes branched but seldom in netlike arrangement. Bundles were never observed. A third component in the shape of rounded particles might have been present, very difficult to distinguish from aggregates of the main component.

It should be pointed out that many of the details now described might be artificial, appearing during the process of desiccation and, therefore, not significant for the natural state of the solutions used for preparation of the objects. For instance, free filaments must always appear in a netlike arrangement, if no forces of orientation come into function during the desiccation. In the present case overlapping of fibres was very seldom observed, the ends being consistently apposed to the neighbouring fibres. This indicates that the net formation was not entirely artificial. Probably a netlike gel was formed when the solution became sufficiently concentrated through evaporation. In the diluted specimen on the other hand, the fibres must have been free.

There is certain evidence to show that the formation of bundles might be entirely artificial. On a number of micrographs from highly purified protein solutions, elsewhere showing pronounced bundle formation, a striking parallel orientation of single fibres was observed. As no signs of tension of the carrier film indicative of an orientation after fixation to the film were visible, it must be assumed that the orientation had taken place during the desiccation process. If the end of a fibre sticks to the carrier film, and the fibre is then caught by the edge of the fluid during evaporation receding from the hydrophobic film, it will be orientated perpendicular to the edge of the fluid. This was probably what had happened in the objects just

mentioned. If this be the case, it indicates that the fibres were not associated to bundles in the solution. The reason why bundle formation appears only in the pure preparations is probably simply that in this case no impurities prevent the complete collapse of the gel structure.

Finally the interpretation of some micrographs as indicative of adsorption of amorphous material on the fibres is of course open to the same criticism. No observations bearing upon the real implication of these pictures were made.

* * *

The sedimentation and diffusion constants of the different preparations indicate a pronounced dissymmetry of the molecules. The numerical values of the dimensions, calculated on the basis of the experimental data, must be considered uncertain and for several reasons. (a) The determination of the constants themselves was not sufficiently accurate. As the dependence upon the concentration, salt content of the solvent, etc. was very marked, the final values had to be estimated through extrapolation, a procedure inevitably involving considerable sources of error. (b) The material was inhomogeneous, and all figures were consequently to be regarded as average values. (c) Finally the method of calculation was valid under certain conditions only. — On that account too much weight must not be attached to the exact figures. Their order of magnitude is, however, significant. Accordingly the particles are to be regarded as slender rods or fibres, probably of a uniform width of 12—13 mμ, their length on the other hand varying considerably. This indicates that the particles are aggregates of smaller units in chainlike association. From the sedimentation data it can be inferred that the tendency to chain formation increases with the degree of purity.

This conception was corroborated by the stream double refraction measurements and above all by the electron optical observations. The estimation of the length of the elementary particles, arrived at micrographometrically, needs further confirmation, however. By application of appropriate methods for preparation of the microscopical objects it might also be possible to study the dissociation phenomenon discussed in chapter IV.

The chemical analysis of the α component is decidedly in favour of the conception of this substance as a nucleoprotein. In the sequel it will be referred to as the high molecular intestinal protein.

Discussion.

The aim of the present study was the isolation in pure state of the viruses of murine and human poliomyelitis. As a working hypothesis it was assumed that these two viruses were closely related, and that in experimental work one of them could serve as model of the other. Consequently all methods were worked out on the mouse virus and then directly applied to the human type.

By means of procedures generally adopted for purification of viruses — with slight modifications necessitated by the nature of the starting material — it was possible to isolate from infected mouse brains a substance, presumably identical with the virus protein. The basic arguments for this presumption were presented in chapter V page 109 and were as follows. (a) The substance was present in all active specimens sufficiently concentrated but absent in concentrates of extracts originating from normal mouse brains. (b) The preparations, representing but 1.5×10^{-4} p. c. of the starting material, had retained about 30 p. c. of the total virus content. Thus the viral activity of the substance was about 200 000 times that of infected brains. (c) The activity of different preparations was proportional to the content of this substance. (d) By sedimentation analysis the purest preparations proved to be homogeneous with a sedimentation constant equal to that of the virus as determined by biological recording.

As previously mentioned the size of the virus as determined by ultrafiltration was estimated to 10—15 mμ, which in case of asymmetric shape of the particles refers to the small axis. In agreement with this figure the molecules of the virus protein were found to be rodshaped or filamentous, the width being 12—13 mμ as determined indirectly from sedimentation and diffusion data or about 15 mμ in electron optical measurements.

In chapter IV the statistical evaluation of titration data led to the conclusion that most of the virus particles appeared in larger aggregates, and that these aggregates probably dissociated on dilution. Sedimentation and diffusion experiments disclosed a certain inhomogeneity of the presumed virus protein, indicating that the particles in reality were aggregates of smaller units associated end to end. This was confirmed in the electron microscope, where the length of the elementary particles was estimated to about 115 mμ. Single virus molecules were scarce, most fibres having a length of about 1 μ, corresponding to 8 or 9 molecules. There was, however, evidence to show that the method for preparation of the dried films favoured aggregation of the particles. It is, therefore, probable that the average of 580 mμ, calculated from the sedimentation experiments, and corresponding to 5 elementary particles, is more correct as an estimate of the length of the chains when in solution. For the same reason it could not be decided whether the aggregation was reversible by dilution.

As developed in chapter IV it must be assumed on anatomical grounds that one MID as a rule comprises more than one active virus particle, aggregated or not. The actual number is dependent upon the relative amount and the distribution of susceptible tissue, which was indicated by the introduction of the resistance factor p. The anatomical structure of the brain and the probable refractoriness of the glial elements considered, one must admit $p = 0.01$, corresponding to 100 particles per MID, to be a plausible value. The molecular weight of the virus protein was calculated to about 57 000 000, referring to aggregates of probably 5 elementary particles, as pointed out above. On the basis of this figure and the last column of table XXII the number of aggregates per MID can be calculated to between 99 and 354.

All these observations give further support to the conception of the substance isolated from infected brains and the filamentous particles observed in the electron microscope as the virus itself.

On account of the minute yields of virus its chemical nature has not yet been studied. In experiment 20 a partially purified preparation was found to contain 0.069 mg nitrogen per ml. This corresponds to a nitrogen content of only 3 p. c. of the dry weight. From the sedimentation diagrams the virus content of this preparation was estimated to about 6 p. c. of the total solids, which calculated

as protein represents 0.023 mg nitrogen per ml. The impurities had obviously a very low nitrogen content, probably consisting largely of lipoids, and the result of the chemical analysis of this specimen, therefore, is not incompatible with the assumption of the virus as a protein.

A comparison with the plant viruses reveals several interesting similarities. Most of these viruses — the only exception so far being that of tomato bushy stunt — are rodshaped with a width of 10—15 mμ and varying lengths. Tobacco mosaic virus is the one best studied. Preparations from young plants purified through differential centrifugation in distilled water are homogeneous with regard to sedimentation with $s_{20} = 174 \times 10^{-13}$ (163). After exposure to salt solutions they become inhomogeneous with a second component of $s_{20} = 200 \times 10^{-13}$. Through viscosity measurements the axial ratio was estimated to 35: 1 on the basis of which the dimensions were calculated to 12.3 × 430 mμ (96). The second component in the sedimentation diagrams corresponds to two such unit particles associated end to end. The diffusion constant was determined to $D_{25} = 0.3 \times 10^{-7}$ (39).

In a recent paper Bernal and Fankuchen (14) reported an extensive study of the X-ray pattern of the virus. In orientated dry gels they found intermolecular spacings of about 150 Å, whereas in wet gels this distance had a minimum value of about 170 Å and varied with pH and salt concentration. They assume that the difference is accounted for by hydration of the molecules.

Kausche, Pfankuch and Ruska (81) measured the particle size on electron micrographs and found a constant width of about 15 mμ, while the length showed a considerable variation. They found no rods shorter than 300 mμ, and most of them held a multiple of this length. A few, however, measured an odd multiple of 150 mμ, indicating that this should be the unit size.

In Wyckoff's centrifugal studies on plant viruses no observations concerning the influence of concentration upon the rate of sedimentation are mentioned. He uses throughout solutions of about 0.15 p. c. virus protein. Likewise no systematical investigation of the dependence upon the ionic strength of the solvent has been carried out. However, from the available data a certain trend of the figures is recognizable (163, 164). The rate of sedimentation seems to be lower in distilled water than in buffer solutions. Furthermore a

certain pH effect is visible, a maximum of sedimentation velocity coinciding with the isoelectric point, with a gradual decrease towards the limits of the stability range. This observation if verified is conform with the result of the X-ray study by Bernal and Fankuchen, and tends to show that the virus is hydrated. This means that the specific volume is larger than the value 0.77, derived from measurements on dried material. The size of the particles, therefore, is probably larger than that estimated by Lauffer. Assuming a degree of hydration as indicated by the X-ray crystallographic measurements one arrives at about 20 × 720 mμ. It is, therfore, very probable, that even the homogeneously sedimenting component of $s_{20} = 174 \times 10^{-13}$ represents aggregates of molecules. The homogeneity might be only apparent, a result of the relatively high concentration of virus. It might be mentioned that the largely inhomogeneous intestinal protein in concentrations of 0.25 p. c. or more in distilled water sediments with abnormally sharp boundaries, the concentration gradients being too steep to be recorded by the scale method.

The electron optical observations on the particle lengths are of considerable interest. Although the existence of unit particles of 150 mμ in length was probable, no such single units were detected. Most aggregates contained an even number of elementary particles, while those with an odd number were scarce. In this connection the dissociation formula C (chapter IV page 76) must be kept in mind. According to that formula even-numbered chains of molecules should predominate if the difference between the two dissociation constants were sufficiently large. From Bald's deductions (4, 5) it is evident that the deviation from the curve of normal distribution of takes is of exactly the same type in tobacco mosaic as in mouse poliomyelitis. In the case of tobacco mosaic, however, there is no way to determine the single molecule lesions and furthermore the probability factor p is of quite different order of magnitude — probably as low as 10^{-6} —, why the dilution endpoint falls in another range of virus concentration. On this account it is not feasible to decide by biological methods whether one or the other dissociation formula is the more probable.

At any rate the neurovirus from mice seems to have many essential qualities in common with tobacco mosaic virus. Most characteristic is the tendency to aggregation of the particles end to end. On the other hand the electron micrographs disclose certain dissimilarities.

Single particles and shorter filaments of the tobacco mosaic virus appear very straight and rigid, whereas the neurovirus seems to be very flexible and elastic, in this respect more like potato X virus (81). This might explain why the tobacco mosaic virus readily forms parallel orientated paracrystals, whereas potato X — and probably the neurovirus — do not.

* * *

When the methods of purification elaborated in experiments on the mouse virus were applied to nervous tissue from cases of human poliomyelitis, small amounts of a substance were recovered, showing a striking resemblance to the mouse virus. This substance was not obtained in pure state — in fact a complete purification was not attempted or even wanted. The yield amounted to a total of about 0.1 mg and as the purification procedure involved separation of the material in substance, it was felt that such small quantities might easily disappear during the manipulations unless some sort of a carrier substance was used. The high molecular weight component present also in extracts of normal nervous tissue served as carrier. It can not be excluded that the presence of this constituent interfered with the rate of sedimentation of the faster component. In preparations of the mouse virus, however, the presence of similar slowly sedimenting material effected no alteration of the sedimentation velocity of the virus. It seems, therefore, justifiable to operate with the values recorded.

The sedimentation constant of this substance was determined to $s_{20} = 150 \times 10^{-13}$, i. e. exactly the same as that of murine virus of the same degree of purity. In the electron micrographs structures of the same shape and size as the mouse virus appeared, embedded in a mass of amorphous material, the carrier substance. Apart from these similarities, the facts that the one preparation tested in a monkey was active, and that the substance now in question was not recovered from normal brains, suggest that it represents the virus protein.

The present study, therefore, has presented further suggestive evidence of the close relationship between the human and the murine poliomyelitis. The working hypothesis, according to which the murine virus and the mouse disease served as model of the human type, has been corroborated.

* * *

The small yield of virus protein obtained from infected nervous tissue necessitated the search for other sources of virus. For this purpose feces from mice and cases of human poliomyelitis, known to contain comparatively large amounts of virus, were most close at hand. As starting material they had certain obvious disadvantages, but were never the less preferable from many points of view. This kind of material would probably be easier to purify; it is available in large quantities — and cheap.

This part of the investigation has hardly passed the preliminary stage. The experimental evidence is still incomplete and in drawing conclusions one must proceed with the greatest caution. In the writer's opinion, however, it offers an interesting point of departure for future work, and it was therefore included in this report.

Before the nature of the high molecular intestinal protein is discussed a few remarks on the purification procedure and the choice of methods should be made. As already mentioned it was observed that tobacco mosaic virus, originally homogeneous, after exposure to salt solutions became inhomogeneous. Parallel with increasing inhomogeneity the rate of sedimentation increased, the stream double refraction indicated increased particle length, the filterability decreased, and the activity decreased. On this account the ammonium sulphate precipitation was regarded as too drastic and inferior to the mild differential centrifugation method.

Bawden and Pirie (8) found that the ability to form liquid crystals depended upon the degree of purity of the virus solution. Bernal and Fankuchen (14) stated that the decisive factor must be the length of the particles, and advanced the theory that low molecular impurities were attached to the ends of the rods, preventing them from associating to longer fibres. As an indirect confirmation of this hypothesis Kausche (80) was able to produce in purified virus solutions real crystals of the same type as those observed in vivo by addition of crude plant juice.

As a corollary of this theory the tendency to aggregation would be a measure of the purity of the solution, and inhomogeneous sedimentation rather to be welcomed than avoided as evidence of successful purification. The effect of salt solutions might be that of elution of impurities adsorbed onto the virus molecules and inseparable from them by mere differential centrifugation. As pointed out on page 124, the experience with the intestinal protein strongly

favours this assumption. The serious problem is whether the treatment with salt has any deleterious effect on the activity. The lesion count is simply a method by which the relative number of infective aggregates is estimated, no matter whether each aggregate contains one or more active virus units. It is therefore quite natural that the activity per unit weight thus measured should decrease with increasing aggregation. This, however, does not necessarily mean that the virus is partially inactivated. The crucial test, reestablishment of the original activity after redispergation of a purified solution, has not yet been carried out.

The incubation time titration method in mouse poliomyelitis is probably independent of the degree of dispersity of the virus. The mouse virus would, therefore, be a suitable test object in experiments concerning the nature of the salt effect. No direct tests to that end were performed, but certain conclusions might be drawn from observations made in purification experiments. The yield of purified virus was about 30 p. c. as estimated from activity tests. In a few cases the different fractions, usually discarded, were systematically sampled and tested for activity. It was found that the loss of activity was approximately accounted for by the total activity of the discarded fractions. The deficit of 70 p. c. was therefore referable to a loss of substance rather than an inactivation of the virus.

Although incomplete as evidence this indicates that the ammonium sulphate precipitation does not damage the virus. The choice of purification method, therefore, should be dependent upon the ultimate aim of the procedure. Treatment with salt solution gives a purer product. If on the other hand preservation of the original physical state rather than chemical purity is wanted, differential centrifugation should be preferred. Concerning these filamentous giant molecules the paradox seems to be applicable, that the more inhomogeneous the purer is the material. It is also obvious that the sedimentation constant is of little value as a characteristic of such substances.

* * *

As described in chapter VI a substance referred to as intestinal protein was regularly found in the feces of man and white mice, regardless whether active virus was excreted or not. It was also recovered from brown rats and pigs but not from white rats and gui-

nea pigs. With the methods used, sedimentation and electron optical analysis, it could not be distinguished from the virus protein. It was composed of molecules or particles of the same size and shape as those of the virus, possibly showing an even more pronounced tendency to aggregation; this, however, could be a secondary phenomenon and the result of a more extensive purification. In active specimens the viral activity was concentrated along with this protein.

From these observations it is evident that it was impossible to decide whether the substance isolated from infective specimens consisted of virus plus inactive protein, or should be regarded as qualitatively homogeneous.

Whichever be the case, it is hardly probable that the similarity between the virus and the "normal" intestinal protein is merely incidental. To the writer's knowledge substances of this type have not previously been isolated from material of animal origin, and those from plants possess viral activity without exception. This field is only just opened, that is true, and but little work has been done as yet.

Claude (27) and later also Furth and Kabat (41, 42, 43, 79) examined heavy fractions obtained by high speed centrifugation of organ extracts. Their preparations were largely inhomogeneous and seemed to consist of cellular debris of particulate nature, probably mitocondria. These particles were, however, of a different order of magnitude when compared with the protein molecules now in question.

Beard and his collaborators (12, 37) when isolating the viruses of rabbit papilloma and equine encephalitis, subjected normal tissue to exactly the same treatment. No substance whatsoever was recovered.

In the process of purification of the neurovirus as well as the intestinal protein a number of heavy components were observed. As revealed by the electron micrographs some of these were particulate, forming spherical bodies of different sizes. Others were finely dispersed, the particle size below the resolving power of the microscope. Similar substances were obtained in control experiments from internal organs such as liver and kidney. However no components of the virus type were detected.

Several virus preparations of animal origin have been examined electron optically but so far no structures similar to those of the intestinal protein have been observed (v. Borries, pers. comm.).

Thus it is evident that substances of this type are at least not common. This circumstance together with the facts that the intestinal protein was recovered from feces of the three species known to contract spontaneously poliomyelitic infections and at least two of them harbouring the virus in their intestines, and also from rats, constant companions of man and pigs, but not from the two further species of animals examined, suggests a closer connection between the virus and this substance than a mere morphological identity.

The assumption that the intestinal protein is an avirulent or non-neurotropic variant of poliomyelitis virus is very tempting. The chemical analysis, showing that it is in all probability a nucleoprotein, supports this hypothesis. The observation that it enhances the activity of a virulent virus, just as do low virulent strains, might have a signification in the same direction. Finally there is some epidemiological evidence in favour of the theory.

From a purely statistical point of departure Petersen (119, 120, 121) has deduced formulas for the development and course of epidemics. His deductions deal with three type cases. (I) The infectious agent is suddenly introduced into a population of a certain average susceptibility. Transmission from one individual to another does not occur. To this type belong for instance the waterborne epidemics of typhoid fever. (II) The infectious agent after being introduced into a population is transmitted from one individual to another through contact, droplet infection or otherwise. Infection produces immunity. (III) The infectious agent is ubiquitous. The individuals are constantly exposed to infection but normally resistant. Under certain conditions a lowering of the general level of resistance occurs, resulting in appearance of disease.

A computation of the epidemic curves out from these assumptions lead to the following results. Type I gives a curve with high initial case incidence, gradually diminishing. Types II and III both give normal distribution curves, provided in the latter case that the individual resistance shows a normal distribution. The study of a single epidemic leaves, therefore, the decision as to type pendent. There are, however, corollaries to the formulas, which make definite conclusions possible after comparative studies of a number of epidemics. In cases of type II the course will be different in intensive and mild epidemics in the same population or in epidemics of the

same average rate of morbidity in large and small populations. Epidemics of type III are independent of these conditions, the only factor affecting the course being the rate at which the resistance changes.

By comparison with the official statistics from different countries Petersen found measles, influenza and other typical contact infections to belong to type II. The agreement between observed and calculated data was excellent. The epidemics of poliomyelitis on the other hand were decidedly of type III. Considering the seasonal distribution and the differences in this respect between the warm and the temperate climates, he searched for a correlation with some well defined climatical factors. He found that the peak of the epidemics occurs at a time of the year when the insolation decreases most rapidly. From the seasonal distribution he calculated the rate of the presumed change of resistance and found a close correlation to the amount of insolation at the corresponding time of the year and the corresponding geographical latitude. His conclusions are, that the virus of poliomyelitis is ubiquitous, and that the lowering of the resistance against infection is the important factor in the epidemiology of the disease. This decrease in resistance coincides with the maximum rate of decrease in the insolation.

The pivotal point of Petersen's deduction is of course the presumed change of the virus-host interaction. For the mathematical treatment it is of no significance whether this change refers to the host, the virus, or both. On the assumption that the decisive factor is increase in virulence of the virus rather than decrease in host resistance, Petersen's theory should be of the utmost importance for the interpretation of the findings reported in this study.

With all reservations for the imperfection of the evidence presented, the following hypothesis will be advanced. The intestinal protein is a virus and a normal inhabitant of the intestines. As to the actual site of infection no conclusive evidence is available. Judging from the experiments on mice the virus is present principally in the contents rather than in the wall of the guts. This suggests, that some intestinal microorganism is the actual host. From the contents virus is probably being continually absorbed by way of the lacteals, deposited in the lymph glands, and gradually destroyed. From this "passive invasion" a slowly increasing serological immunity follows, a process that is probably intensified by intercurrent infections,

which would explain why the neutralizing substances develop earlier in urban than in rural populations.

Normally this virus is non-neurotropic, and as it does not possess avidity to any other tissues of the organism, its presence can not be revealed by our present biological tests. From a pathological point of view it must be characterized as not only apathogenic but non-infectious. Under certain conditions, however, the formation of neurotropic variants is favoured. In the domesticated mouse, not exposed to any considerable seasonal changes of the environment, these conditions are established, when the animals are being weaned, no matter what season. In man they are somehow, directly or indirectly, connected with certain climatic factors.

The nature of the active forces and the mechanism of the process can be but conjectured. It is well known that certain external factors like radiation, chemical treatment, etc. favour the appearance of mutants. If the intestinal virus were confined to the intestines of the respective hosts the active agent causing mutation most probably would have to be introduced with the food. If on the other hand an intestinal microorganism is the real carrier of the virus, this vector could be supposed capable also of living outside its host, and thus to be exposed more directly to the hypothetical agent. Whatever the mechanism water would be the most probable vehicle. There already exists some experimental and epidemiological evidence to that effect. A transmissible agent, causing poliomyelitis in monkeys, was recovered by Kling (86) from suspected well water. Furthermore the connection between athmospheric precipitation and poliomyelitis (45) suggests that surface polluted drinking water be a vehicle of the virus or the transformation agent.

When the suitable conditions cease to exist, the neurotropism subsides, either through disappearance of the mutant strains or through a gradual decrease in affinity to nervous tissue. In favour of the latter alternative is the common experience, that newly isolated strains of virus often cannot be established in passage at all, or they display a gradual decrease in virulence, extinguishing completely after 3 or 4 passages. In some strains, however, the neurotropism becomes fixed, making the individual host a carrier for life.

According to this theory the poliomyelitic infection would be in a sense endogenic, although certain exogenic factors are involved as well. There are other virus infections that could be explained on a

similar basis. In connection with herpes for instance the endogenic origin of virus has been most ardently discussed. In disposed persons the herpes eruptions can be produced at will by various stimulants, principally fever producing agents. Virus can be regularly recovered from the vesicles. In the meantime between attacks, on the other hand, it is usually not possible to detect the virus anywhere (for references see Doerr (30)). And yet it must be present in the tissues permanently. — Shope (138) produced conclusive evidence to show that the virus of swine influenza is transmitted by the lungworm, the eggs of which are excreted with the feces and taken up by earthworms. These in their turn are devoured by the swine, the lungworm develops in the intestines, penetrates the wall, wanders to the lungs, and the cricle is shut. That the virus is transmitted by this route is proved beyond doubt. Yet it has not been possible to show the existence of the virus in the lung worm in any stage of its developmental cycle. This must mean that the virus does not develop pneumotropic properties until the vector has reached the lungs of the host. As the pathogenicity of the virus is the only characteristic by which it can be biologically identified the non-pneumotropic variant cannot be detected.

Another interesting parallel can be drawn. The investigations by Claude (25, 26, 27) have revealed that the tumor producing agent from Rous' sarcoma is inseparably connected with certain particulate structures in the tumor extracts. The size of these particles is in the range of 50—200 mμ. They were found to consist of a nucleoprotein and about 50 p. c. phospholipoids. However, particles of the same size and the same chemical composition were recovered from normal tissues as well. To these particles are attached the heterogenetic antigen (41), the Wassermann antigen (42), and certain enzymes (79). They are regarded by Claude as preformed cellular structures, probably mitochondria. — In this case it is hardly appropriate to denote the particulate component of the tumor extracts as the virus of Rous' sarcoma. The tumor producing agent is combined with the mitochondria and its activity obviously intimately connected with the maintenance of the structure of these particles. Although pathologically altered they are nevertheless cellular constituents and might perhaps be regarded as mutant mitochondria.

The virus of poliomyelitis, on the other hand, is not a constituent of the cell. No similar substance or structure is present in normal

cells. However, the theory cannot be worked out in detail at present. The supporting evidence has obvious defects, and consequently the next objects of research are given: the chemical and serological relation between the neurovirus and the high molecular intestinal protein, the origin of the latter substance, and eventually the conditions necessary for its transformation into a neurotropic variant.

Summary.

The aim of this study was the purification of the viruses of mouse encephalomyelitis (Theiler's disease) and human poliomyelitis.

Chapter I contains a review of the systematical position of the viruses in question. The virus of encephalomyelitis enzootica suum (the Teschen disease) is also included in the survey. The similarities between these three viruses are stressed. On the basis of the discussion a more uniform nomenclature is suggested, using the name poliomyelitis virus as species denomination, with human, murine, and porcine strains as subspecies or types.

The second chapter is an account of the general methods applied and the material used. Attempts at tissue culture of the mouse virus were without success.

In chapter III the methods for measurement of virus activity are reviewed. It was shown that the mouse virus can be propagated serially in the developing chick. The infection was restricted to the central nervous system, the final virus concentration remained on a low level, and no characteristic lesions were produced. The membrane technique, therefore, was not applicable to poliomyelitis virus.

The incubation time titration method was studied with special regard to the sources of error. The dependence of the incubation period upon the amount of virus inoculated, the volume of the inoculum, the weight, sex and age of the test animals was analysed. A linear relation was shown to exist between the reciprocal of the harmonic mean of the incubation time and the logarithm for the dilution. The slope of the titration curve was dependent upon the volume of the inoculum and the age of the test animals. Finally the accuracy of the method was estimated and its field of application discussed.

The accuracy of the 50 p. c. endpoint method and the relation of the titer-values to those obtained with the former method was analysed.

In chapter IV the theoretical basis for the interpretation of titration data is surveyed. Parker's experiments on the virus of vaccinia are discussed and a different interpretation suggested, based upon the assumption, that the virulence of a certain strain of virus is connected with the presence in the elementary bodies of an activator, necessary for the incitement of infection.

An identical experiment was carried out with the FA strain of mouse poliomyelitis virus. An analysis of this experiment showed that a group of animals could be recognized, in which the infection was probably incited by a single virus molecule. This observation formed a basis for the further discussion, which led to the conclusion that the virus must appear largely as aggregates of elementary particles, and that this aggregation might be reversible on dilution.

The same observation was very valuable as a point of departure in attempts to explain the connection between the amount of virus inoculated and the ensuing incubation period. It was assumed that an inhibitory influence upon the multiplication of the virus is exerted by intact ganglion cells and also transmitted to neighbouring cells. The degree of this cellular resistance against infection might be connected with the intensity of metabolism of the cell.

Chapter V gives a description of the purification of murine and human virus from brain and spinal cord. Through a combination of salting-out and differential centrifugation, preparations were obtained from infected mouse brains, the activities of which on a weight-by-weight basis were up to 200 000 times that of the starting material. In sedimentation analysis all preparations concentrated above a certain limit showed the presence of a component with a sedimentation constant of the order of 150—160 $\times 10^{-13}$. No such constituent was recovered from normal brains. The purest preparations were homogeneous in the ultracentrifuge. The rate of sedimentation increased with the degree of purity and was higher in salt solutions than in distilled water. The sedimentation constants recorded were: less pure material in distilled water $s_{20} = 150 \times 10^{-13}$, pure material in distilled water $s_{20} = 162 \times 10^{-13}$, in 0.05 m sodium chloride $s_{20} = 181 \times 10^{-13}$.

The sedimentation of the virus was studied in the separation cell by means of activity measurements. The sedimentation constant of the biologically active component was determined to $s_{20} = 152 \times 10^{-13}$.

The titration data revealed the presence in crude brain extracts of inhibiting substances not combining with the virus in vitro. They were destroyed by digestion with pancreatic juice. As they were not present in extracts of normal brain they might have some connection with the tissue immunity.

When spinal cords from cases of human poliomyelitis were purified according to the same method, small amounts of a similar component were obtained, which was not present in normal brain and spinal cord identically treated. The sedimentation constant was determined to $s_{20} = 150 \times 10^{-13}$.

The yield of substance averaged 0.6 γ per mouse brain and a total of 0.1 mg from 11 human spinal cords.

In chapter VI the purification of feces and intestinal contents is described. The method of purification was essentially the same as that applied to nervous tissue. From human stools, preparations consisting of three main components were obtained. One of these resembled closely the substance obtained from infected nervous tissue. Its rate of sedimentation was dependent upon its concentration and the salt content of the solution. The limit value of the sedimentation constant was estimated to $s_{20} = 165 \times 10^{-13}$ in distilled water, and $s_{20} = 220 \times 10^{-13}$ in 0.05 m sodium chloride solution. A certain amount of impurities, probably attached to the main component in the form of an adsorption compound could not be removed through differential centrifugation in distilled water or dilute sodium chloride solution, but was eliminated by treatment with 0.4 m ammonium sulphate. A pure preparation of this component had retained presumably all of the original virus activity.

However, an identical substance could be isolated not only from all cases of poliomyelitis examined, but from healthy contacts and from normal controls as well. The yield was practically constant, about 1 mg per 100 g of solids, regardless of the origin of the specimen.

In feces from normal mice a component of the same type was present. The yield corresponded to an excretion of 0.01 mg per mouse and day. The pure preparations from young animals produced typical poliomyelitis when inoculated intracerebrally, the activity being 100—1000 times that of the starting material. The substance derived from old animals was inactive even if inoculated in large quantities.

The purified intestinal virus showed no interference phenomenon when inoculated together with the virulent encephalitogenic FA strain. Addition of the intestinal virus increased the activity of a FA preparation as would an additional amount of the FA virus itself. Preparations from old animals, in themselves inactive, had a similar, although less pronounced, enhancing effect upon the FA strain.

Feces from a number of other animal species were examined for the presence of similar substances. No such components were obtained from guinea pigs and white rats. Small quantities of a presumably identical substance were detected in the intestinal contents from three pigs, and large amounts were constantly recovered from the feces of wild brown rats. Repeated tests for activity of these preparations in monkey, brown rats, and white mice were negative.

In chapter VII some experiments on purified material are reported. The diffusion constant of the substance derived from infected mouse brains was determined to $D_{20} = 0.32 \times 10^{-7}$. The intestinal components from man, the white mouse, and the brown rat were found to have the same diffusion constant $D_{20} = 0.11 \times 10^{-7}$. On the basis of these values and the corresponding sedimentation constants, and on the assumption of cylindrical and non-hydrated molecules the following characteristics were computed. Material from nervous tissue: molecular weight 57×10^6, particle size 12.5×580 mμ. Intestinal material: molecular weight 200×10^6, particle size 12.2×2150 mμ.

A pure preparation of intestinal material of human origin was examined for ultraviolet absorption. It showed a relative maxium of absorption in the range of 2600—2700 Å and a practically complete absorption of wavelengths below 2400 Å. A chemical analysis of the same preparation gave a nitrogen content of 16 p. c. and a phosphorus content of 0.6 p. c. Thus, the substance was probably a nucleoprotein.

In immunization experiments on rabbits, the intestinal proteins proved to be antigenically active and specimens from man, the white mouse, and the brown rat showed serological relationships.

Electron optical observations on the purified materials revealed the presence in all specimens examined of filamentous structures of uniform appearance. The width of the fibres was estimated to about 15 mμ. The length varied considerably and seemed to be

dependent to a certain extent upon the state of purity of the specimen. The purer the material, the longer the fibres. Simultaneously a tendency to aggregation side by side could be detected. Through a statistical evalution of the particle lengths it was assumed that the fibres were composed of a varying number of smaller units, the length of the elementary particles being estimated to about 115 mμ.

In chapter VIII the results are summarized and discussed. On the basis of the discussion it is concluded that the substance obtained from infected mouse brain must be regarded as the pure virus protein. A comparison with the plant viruses reveals many interesting similarities.

Likewise the corresponding component of preparations from spinal cords of human cases of poliomyelitis probably represents the human virus protein. The investigation has further corroborated the assumption of the close relationship between the human and the murine virus.

The purification methods are discussed. The salting-out procedure gives a purer product. The differential centrifugation preserves the original physical state of the material, but gives a less pure product.

In a discussion of the nature of the high molecular intestinal protein it is pointed out that final conclusions cannot be drawn, as the evidence is still incomplete. A theory is advanced according to which this substance is regarded as a virus, ordinarily non-neurotropic but under certain conditions developing neurotropic properties.

Appendix.

1) *Hospital record numbers* of patients from which material was collected. The letters denote: A = Medical School Hospital, Uppsala; B = Hospital of Epidemic Diseases, Bollnäs; G = Hospital of Epidemic Diseases, Gävle; S = Hospital of Epidemic Diseases, Stockholm; U = Hospital of Epidemic Diseases, Uppsala.

Brain and spinal cord	Fæces		
	Poliomyelitis cases		Controls
A 1722/41	S 2674/41	U 259/42	A 179/42
U 201/41	S 2685/41	U 260/42	A 226/42
	S 2686/41	U 261/42	A 378/42
B 121/42	S 2692/41	U 264/42	A 452/42
B 126/42	S 2696/41	U 265/42	A 464/42
B 133/42	S 2714/41	U 272/42	A 482/42
B 147/42	A 24/42	U 273/42	A 526/42
B 152/42	A 340/42	U 274/42	A 555/42
B 153/42	A 413/42	U 277/42	A 572/42
G 190/42	A 512/42	U 283/42	A 613/42
G 196/42	G 174/42	U 284/42	A 629/42
S 1893/42	G 179/42	U 288/42	A 670/42
U 329/42	G 180/42	U 291/42	A 671/42
	G 182/42	U 292/42	A 707/42
	G 184/42	U 297/42	A 1277/42
	G 185/42	U 301/42	A 1724/42
	G 207/42	U 319/42	A 1725/42
	G 209/42	U 320/42	A 1753/42
	G 211/42	U 325/42	A 1786/42
	G 214/42	U 332/42	A 2001/42
	G 216/42	U 344/42	A 2458/42
	G 217/42	U 346/42	A 2474/42
	U 252/42	U 347/42	
	U 253/42	U 363/42	
	U 258/42		

Material was collected from one further lethal case of poliomyelitis, the father of patient B 147/42, delivered to the Province Hospital, Bollnäs, but never filed in the records. Sections of the spinal cord from this case were examined microscopically and showed typical poliomyelitic lesions.

2) *Formulas used.*

Chapter III, page 55. Quotient of two non-correlated means

$$M = \frac{M_1}{M_2} + \frac{M_1}{M_2^3} \cdot \varepsilon(M_2)^2.$$

Standard error of quotient

$$\varepsilon(M) = \pm \sqrt{\frac{\varepsilon(M_1)^2}{M_2^2} + \frac{M_1^2}{M_2^4} \cdot \varepsilon(M_2)^2}.$$

Chapter VII, page 134. Correction of sedimentation constant to density and viscosity of water

$$s_{corr} = s_{obs} \cdot \eta \cdot \frac{1 - V \cdot 0.9982}{1 - V \cdot \varrho}$$

η being viscosity of solvent, ϱ specific gravity of solvent and V specific volume of the substance.

Concerning the formulas for calculation of molecular weight, frictional ratio, and axial ratio, the reader is referred to T. Svedberg and K. Pedersen: The Ultracentrifuge (146 a).

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PLATES

I—III

Plate I

Mouse poliomyelitis

a) Animal experimentally infected with strain UF I. Partial paralysis of the hind legs.

b) Section of brain cortex. Perivascular cuffing. (Strain UF I.) Magnification 100x.

c) Section from the region of the fourth ventricle. Perivascular cuffing, glial reaction and round cell infiltration, neuronophagia. Plexus chorioideus intact. (Strain UF I.) Magnification 100x.

d Section of spinal cord. Perivascular cuffing and round cell infiltration in anterior horn. (Strain UF I). Magnification 100x.

e) Section of spinal cord. Neuronophagia. (Strain UF I.) Magnification 900x.

f) Section of spinal cord. Perivascular cuffing, scattered infiltration, neuronophagia. (Strain FA.) Magnification 100x.

g) and h) Animal experimentally infected with strain FA. Fit of »jitter-bug disease», clonic convulsions.

i and k) Sections of brain cortex. Intense reaction with perivascular cuffing and scattered infiltration. (Strain FA.) Magnification 100x.

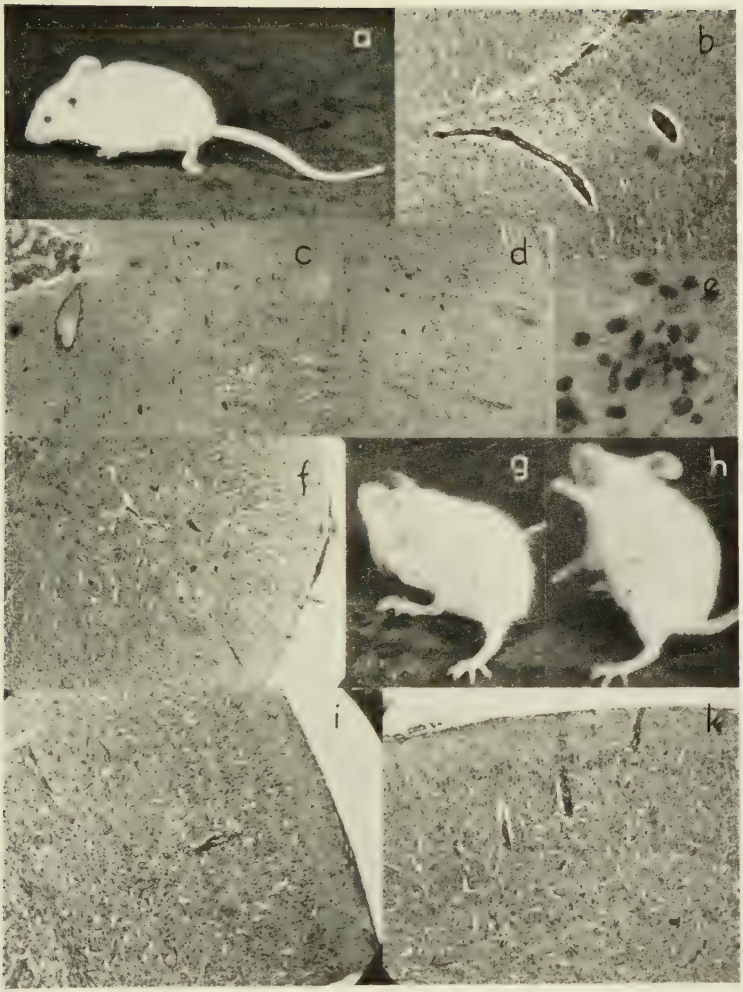
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Plate II

Monkey poliomyelitis

a) MC 1. Section of spinal cord. Magnification 70x.
b) MC 1. Section of spinal cord. Neuronophagia. Magnification 900x.
c) and d) MR 5. Sections of spinal cord. Magnification 100x.
e) MR 8. Section of spinal cord. Magnification 70x.
f) MR 8. Section of spinal cord. Neuronophagia. Magnification 900x.

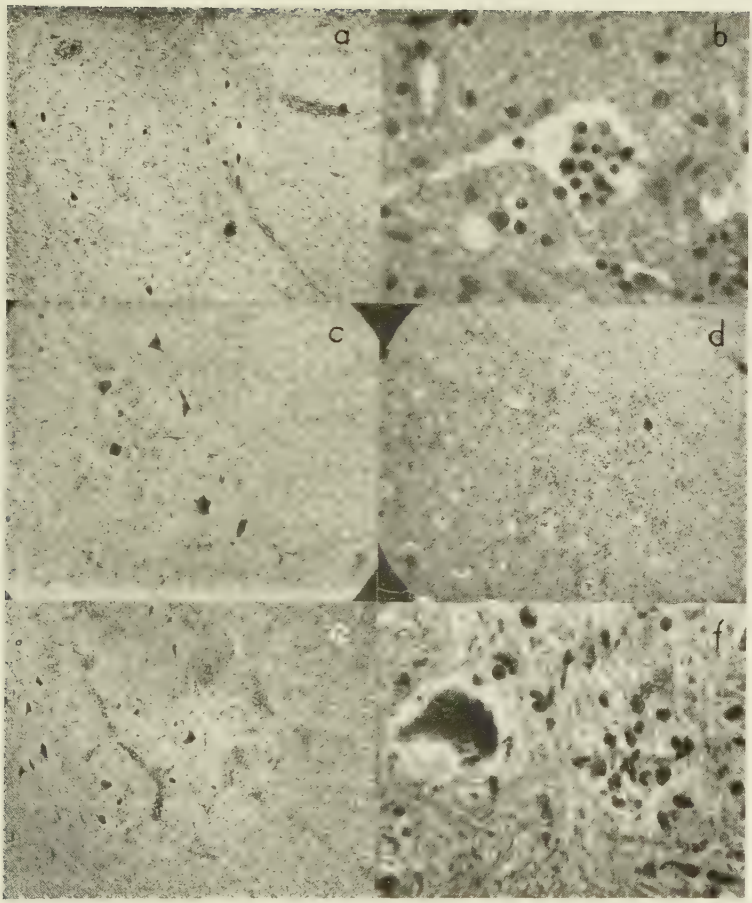
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Plate III

with permission of the Siemens und Halske A.-G. quoted from Gard: Arkiv för Kemi Bd **17 B,** no. 1 (cf. 49).

a) Neurovirus from mice. Impurities attached to the fibres. Electron optically 22000: 1.

b) Neurovirus from man. The finely dispersed »carrier substance» blurring the outlines of the fibres. Electron optically 24000: 1.

c) Intestinal protein from mice. Gel structure in concentrated material. Electron optically 25000: 1.

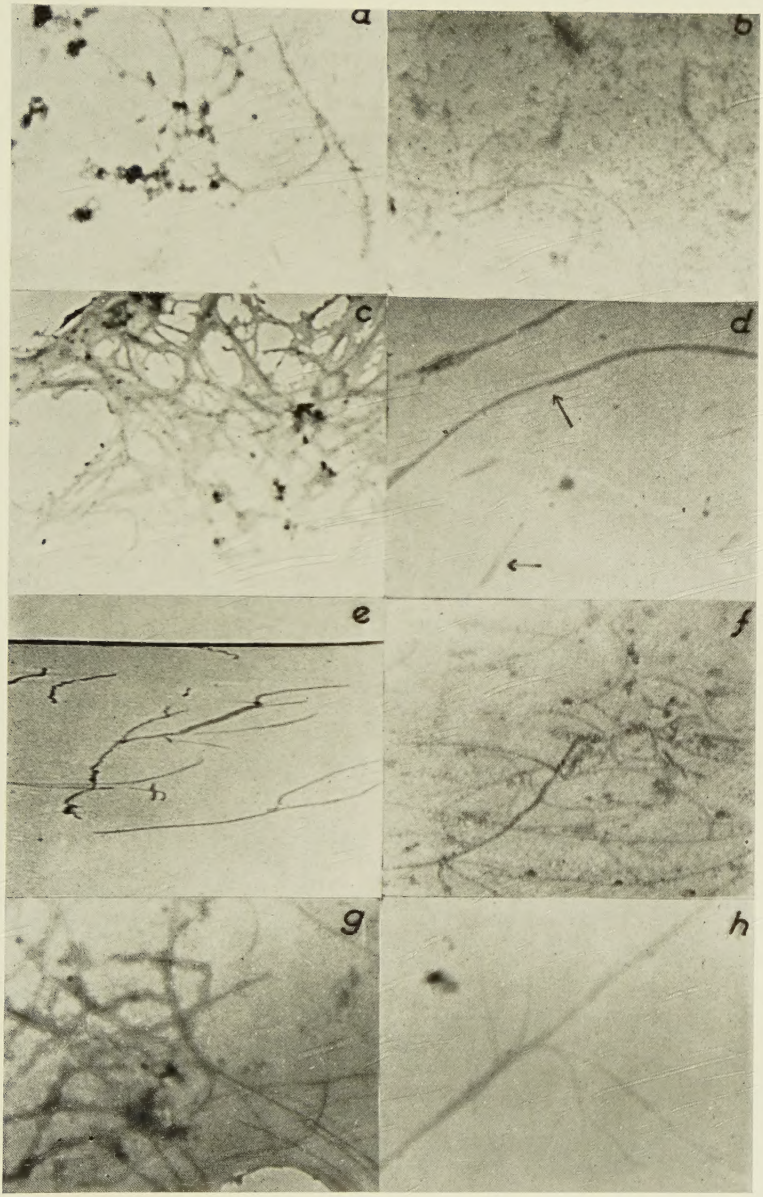
d) Intestinal protein from mice. Bundle formation. The arrows indicate ends of individual fibres. Scattered single and double molecules visible. Electron optically 23000: 1.

e) Intestinal protein from mice. Carrier film disrupted, filaments under tension largely deformed. Electron optically 23000: 1.

f Intestinal protein from man. Impure. Particulate matter enmeshed in the network. Electron optically 27000: 1.

g Intestinal protein from man. Medium purity. Low molecular material adsorbed on the fibres. Electron optically 25000: 1.

h Intestinal protein from man. Highly purified. Bundle formation. Electron optically 22000: 1.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